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Dan Weng

*University of Massachusetts Medical School*

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**CASPASE-8 AND RIP KINASES REGULATE  
BACTERIA-INDUCED INNATE IMMUNE  
RESPONSES AND CELL DEATH**

A Dissertation Presented

By

**Dan Weng**

Submitted to the Faculty of the

University of Massachusetts Medical School of Biomedical Sciences, Worcester in  
partial fulfillment of the requirements for the degree of

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JULY 7, 2014

INTERDISCIPLINARY GRADUATE PROGRAM

**CASPASE-8 AND RIP KINASES REGULATE BACTERIA-INDUCED INNATE  
IMMUNE RESPONSES AND CELL DEATH**

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## Abstract

*Yersinia pestis* (*Y. pestis*), as the causative agent of plague, has caused deaths estimated to more than 200 million people in three historical plague pandemics, including the infamous Black Death in medieval Europe. Although infection with *Yersinia pestis* can mostly be limited by antibiotics and only 2000-5000 cases are observed worldwide each year, this bacterium is still a concern for bioterrorism and recognized as a category A select agent by the Centers for Disease Control and Prevention (CDC). The investigation into the host-pathogen interactions during *Y. pestis* infection is important to advance and broaden our knowledge about plague pathogenesis for the development of better vaccines and treatments.

*Y. pestis* is an expert at evading innate immune surveillance through multiple strategies, several mediated by its type three secretion system (T3SS). It is known that the bacterium induces rapid and robust cell death in host macrophages and dendritic cells. Although the T3SS effector YopJ has been determined to be the factor inducing cytotoxicity, the specific host cellular pathways which are targeted by YopJ and responsible for cell death remain poorly defined. This thesis research has established the critical roles of caspase-8 and RIP kinases in *Y. pestis*-induced macrophage cell death. *Y. pestis*-induced cytotoxicity is completely inhibited in RIP1<sup>-/-</sup> or RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> macrophages or by specific chemical inhibitors. Strikingly, this work also indicates that macrophages deficient in either RIP1, or caspase-8 and RIP3, have significantly reduced infection-induced production of IL-1 $\beta$ , IL-18, TNF $\alpha$  and IL-6 cytokines; impaired activation of NF- $\kappa$ B signaling pathway and greatly compromised caspase-1 processing; all of which are critical for innate immune responses and contribute to fight against pathogen infection. *Y. pestis* infection causes severe and often rapid fatal disease before the development of adaptive immunity to the

bacterium, thus the innate immune responses are critical to control *Y. pestis* infection. Our group has previously established the important roles of key molecules of the innate immune system: TLR4, MyD88, NLRP12, NLRP3, IL-18 and IL-1 $\beta$ , in host responses against *Y. pestis* and attenuated strains. *Yersinia* has proven to be a good model for evaluating the innate immune responses during bacterial infection. Using this model, the role of caspase-8 and RIP3 in counteracting bacterial infection has been determined in this thesis work. Mice deficient in caspase-8 and RIP3 are very susceptible to *Y. pestis* infection and display reduced levels of pro-inflammatory cytokines in spleen and serum, and decreased myeloid cell death. Thus, both *in vitro* and *in vivo* results indicate that caspase-8 and RIP kinases are key regulators of macrophage cell death, NF- $\kappa$ B and caspase-1 activation in *Yersinia* infection. This thesis work defines novel roles for caspase-8 and RIP kinases as the central components in innate immune responses against *Y. pestis* infection, and provides further insights to the host-pathogen interaction during bacterial challenge.

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## List of Publications

**Dan Weng**, Robyn Marty-Roix, Sandhya Ganesan, Megan K. Proulx, Gregory I. Vladimer, William J. Kaiser, Edward S. Mocarski, Kimberly Pouliot, Francis K.-M. Chan, Michelle A. Kelliher, Phillip A. Harris, John Bertin, Peter J. Gough, Dmitry M. Shayakhmetov, Jon D. Goguen, Katherine A. Fitzgerald, Neal Silverman and Egil Lien. **Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death.** Proc Natl Acad Sci USA. 2014 May 20;111(20):7391-6.

Vladimer GI, **Weng D**, Paquette SW, Vanaja SK, Rathinam VA, Aune MH, Conlon JE, Burbage JJ, Proulx MK, Liu Q, Reed G, Meccas JC, Iwakura Y, Bertin J, Goguen JD, Fitzgerald KA, Lien E. **The NLRP12 inflammasome recognizes *Yersinia pestis*.** Immunity. 2012 Jul 27;37(1):96-107.

Dan Weng generated data shown in Figure S1C, contributed to *in vivo* experiments and purified lipids from *Yersinia pestis* for Figure S1AB.

Vladimer GI, Marty-Roix R, Ghosh S, **Weng D**, Lien E. **Inflammasomes and host defenses against bacterial infections.** Curr Opin Microbiol. 2013 Feb;16(1):23-31.

Dan Weng wrote the section on cell death after inflammasome activation.

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## **CHAPTER I: Introduction**

## **Innate Immune response**

The host innate immune system is the first protective line of defense against invading pathogens. Upon pathogenic organism infection, the host instantly initiates various innate immune responses to restrict the pathogen growth as well as guide the adaptive immune system. These innate responses include phagocytosis, microbial killing, and the synthesis of pro-inflammatory cytokines and chemokines that induce inflammation and recruit other immune cells. Pattern recognition receptors (PRRs), which are germ-line encoded, constitute important innate immunity components. PRRs sense extracellular or intracellular microorganisms through recognizing conserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Janeway and Medzhitov, 2002; Kawai and Akira, 2010). Once activated, PRRs initiate an intracellular signaling cascade, culminating in the activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B), MAP kinases (MAPK) and interferon regulatory factor (IRF) families of transcription factors, which are responsible for the production of immunomodulatory cytokines, chemokines or interferons (Janeway and Medzhitov, 2002; Kawai and Akira, 2010). Beyond the classical innate immune responses mentioned above, growing evidence implicates that pathogen infection-induced host cell death emerges as another mechanism with particular impact on the innate immune system. Apoptosis, necrosis as well as pyroptosis constitute three distinct modes of cell death, and all of them are closely involved in the host immune response to microbial infection (Ashida et al., 2011; Lamkanfi and Dixit, 2010).

## **Toll-like receptors**

Pattern recognition receptors (PRRs) are a large group of extracellular and intracellular receptors

that recognize unique PAMPs and DAMPs. PAMPs are molecular patterns specific to pathogens, while DAMPs are endogenous molecules released from damaged cells. PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), C-type lectin receptors (CLRs) and AIM2-like receptors (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Among them, the TLR family is one of the best-characterized PRR families. The TLRs are type I integral membrane receptors, which are composed of N-terminal leucine-rich repeats (LRRs), a transmembrane helix region and a C-terminal cytoplasmic Toll-interleukin receptor (TIR) domain (Bell et al., 2003; Kumar et al., 2011). The horseshoe-shaped LRR domains are responsible for recognizing TLR-specific ligands. The binding of the TLR ligands result in dimerization of the LRR domains, which bring the TLRs' transmembrane and cytoplasmic TIR domains in close proximity and trigger a downstream signaling cascade by recruiting the adaptor proteins. There are five adaptors downstream of TLRs, including MyD88 (myeloid differentiation factor 88) (Medzhitov et al., 1998; Muzio, 1997; Wesche et al., 1997), Mal (MyD88 adaptor like protein, also called TIRAP) (Fitzgerald et al., 2001), TRIF (TIR domain containing adaptor inducing interferon- $\beta$ ) (Yamamoto et al., 2003a), TRAM (TRIF related adaptor protein) (Fitzgerald et al., 2003; Hoebe et al., 2003; Yamamoto et al., 2003b), and SARM (sterile- $\alpha$  and HEAT/Armadillo motifs-containing protein) (Carty et al., 2006).

Currently, there are 12 TLRs identified in mice and 10 in humans. TLR1 to TLR9 are conserved in both species. Mouse TLR10 is a pseudogene and not functional. TLR11, TLR12 and TLR13 have been lost from the human genome (Kawai and Akira, 2010). Different TLRs sense different conserved molecular patterns of microorganisms and self-components. These TLRs can be largely divided into two categories based on their cellular locations, TLR1, 2, 4, 5, and 6 are



expressed and located on the cell plasma membrane and recognize pathogen membrane components like lipids, lipoproteins and proteins. TLR3, 7, 8, 9, 11 and 13 are expressed in intracellular endosomal compartments and largely recognize nucleic acids (Kawai and Akira, 2011). Specifically, TLR2 interacts with either TLR1 or TLR6 to recognize various PAMPs from bacteria, mycoplasma, fungi, and viruses, including bacterial lipopeptides and peptidoglycan, zymosan from fungi, etc (Takeuchi and Akira, 2010). Typical ligands for the TLR2-TLR1 heterodimer are triacylated lipopeptides (Pam3Cys4) from Gram-negative bacteria and mycoplasma, while diacylated lipopeptides (Pam2Cys4) from Gram-positive bacteria are the typical ligands for the TLR2-TLR6 heterodimer (Kawai and Akira, 2010). TLR5 recognizes flagellin, a protein component of bacterial flagella. *Tlr10* is a pseudogene in mice due to sequence gap and multiple retroviral insertion (Hasan et al., 2005). The role of TLR10 in humans is still unclear and it remains an orphan receptor without a known agonist or function. Recent studies suggest that TLR10 expression can be up-regulated by influenza virus and TLR10 contributes to the sensing of influenza viral infection, but the exact ligand for TLR10 is yet to be identified (Lee et al., 2014). TLR11 has close homology to TLR5 and is expressed in mice but not in humans. TLR11 senses uropathogenic bacteria and, in cooperation with TLR12, binds to a profilin-like molecular pattern from the intracellular protozoan *Toxoplasma gondii* (Yarovinsky et al., 2005). TLR13 has been recently identified to recognize bacterial ribosomal RNA (Hidmark et al., 2012; Oldenburg et al., 2012). TLR3, 7, 8, 9 are intracellular TLRs and are mainly located in the endosomal compartment. While TLR3 is known to recognize dsRNA, TLR7 and 8 sense ssRNA. TLR9 detects unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) CpG motifs, which are frequently found in bacteria but rare in vertebrates. To mimic these natural ligands, poly (I:C) (a synthetic dsRNA) is often used as a TLR3 ligand, while synthetic CpG DNA is used as a TLR9

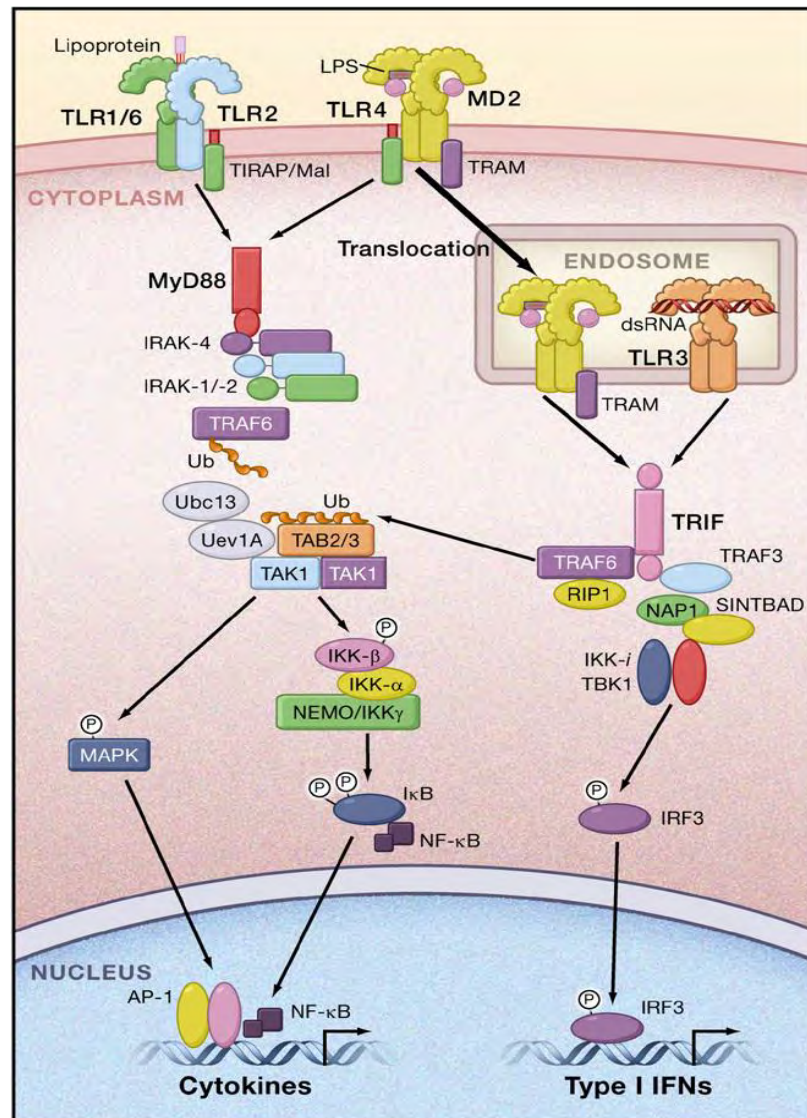
ligand, and the imidazoquinoline compounds such as R848 as TLR7/TLR8 ligands in experiments.

### **Toll-like receptor 4 (TLR4)**

TLR4 is probably the most extensively characterized toll-like receptor. It recognizes lipopolysaccharide (LPS) together with the adaptor myeloid differentiation factor 2 (MD2) (Medzhitov et al., 1997; Shimazu et al., 1999). LPS is the major component of Gram-negative bacterial cell-wall, and is also known as endotoxin, the agent responsible for septic shock. LPS is composed of a lipid A portion anchored in the outer membrane, a polysaccharide core structure and an O-antigen (Raetz and Whitfield, 2002). The lipid A of LPS is the part recognized by TLR4-MD2 complex. The crystal structure of the TLR4-MD2-LPS complex indicates that two TLR4-MD2 complexes interact symmetrically to form a homodimer via the hexa-acyl-chains of lipid A, which are embedded in the hydrophobic pocket of MD2 or is hydrophobically bound to TLR4 (Park et al., 2009). The dimerization of the TLR4/MD2 complex initiates the signaling cascade by recruiting the adaptor proteins to its TIR domains. TLR4 is the only TLR which uses all four adaptor proteins (MyD88, Mal, TRIF and TRAM) to mediate its immune signaling pathway, and it can be divided into MyD88-dependent and MyD88-independent pathways (Fig 1.1) (Takeuchi and Akira, 2010; Yamamoto et al., 2003b).

Activation of the MyD88-dependent pathway leads to a fast, robust early activation of NF- $\kappa$ B and MAPKs. Upon LPS binding to TLR4/MD2, Mal and MyD88 are recruited to the TIR domain of TLR4 at the plasma membrane. Then IRAK (IL-1 receptor-associated kinase) family members are engaged to MyD88/Mal. The first recruited and activated one is IRAK4, followed by IRAK1

and IRAK2 (Fig. 1.1). TRAF6 (tumor necrosis factor receptor-associate factor 6) subsequently interacts with the IRAKs, leading to TRAF6's phosphorylation and activation. TRAF6 acts as an E3 ligase to add K63 (Lys63)-linked polyubiquitin chains to target proteins, including TAK1 (transforming growth factor- $\beta$ -activated kinase 1), TAB2, TAB3 and NEMO (I $\kappa$ B kinase (IKK) subunit NF- $\kappa$ B essential modifier, also called IKK $\gamma$ ). Activated TAK1 phosphorylates IKK $\beta$ , which leads to the phosphorylation, ubiquitination and degradation of I $\kappa$ B (inhibitor of  $\kappa$ B). I $\kappa$ B degradation frees NF- $\kappa$ B and allows it to translocate into nucleus, leading to the transcription of pro-inflammatory cytokines and antimicrobial factors (Fig. 1.1). TAK1 also activates the MAPKs, including ERK, p38 and JNK, which trigger the signal transduction, resulting in the activation of transcription factors, like AP-1 (Takeuchi and Akira, 2010) (Fig. 1.1). MyD88 is required by all of the TLRs (except TLR3) to implement their effects. TLR3, however, utilizes MyD88-independent TRIF-dependent pathway to realize its pro-inflammatory effects. The TRIF/TRAM-dependent pathway acts as the other arm of signaling downstream of TLR4, mediating the activation of late phase NF- $\kappa$ B and IRF pathways. After initiation of MyD88-mediated pathway, TLR4 moves from the plasma membrane to the endosomes and activates the TRIF/TRAM-dependent pathway (Fig. 1.1) (Kawai and Akira, 2010). At the endosome, TRIF and TRAM are recruited to the TIR domain of TLR4. Subsequently, TRIF recruits RIP1 (Receptor interacting protein 1) through its RHIM (RIP homotypic interaction motif) domain, as well as TRAF6 and TRADD, leading to the activation of TAK1, which in turn activates the NF- $\kappa$ B pathway. Additionally, TRIF also recruits and activates TRAF3, which causes the formation of a signaling complex composed of the noncanonical IKKs TBK1 and IKK $\epsilon$  (also called IKK-i). TBK1 and IKK $\epsilon$  then catalyze the phosphorylation of IRF3 and allow its nuclear translocation, leading to the transcription and production of Type I interferons (IFN $\alpha$  and IFN $\beta$ ) (Fig. 1.1) (Kawai and Akira, 2010; Takeuchi and Akira, 2010).



Takeuchi and Akira, Cell. 2010 Mar 19;140(6):805-20.

**Figure 1.1 Schematic representation of TLR4, TLR2 and TLR3 signaling pathways**

LPS recognized by TLR4/MD2 initiates both MyD88-dependent and MyD88-independent pathways. dsRNA recognized by TLR3 only initiates TRIF-mediated pathway while lipoprotein by TLR1/6 and TLR2 leads to MyD88-dependent pathway. MyD88 and Mal are recruited to the TLR, and subsequently IRAKs and TRAF6 are also recruited. TRAF6, together with an E2 ubiquitin ligase complex of Ubc13 and Uev1A, catalyze a K63-linked polyubiquitin chain on TRAF6 itself and the TAK1-TAB2/3 complex, which leads to the activation of NF-κB and MAPK pathways. At the endosome, TRAF6, RIP1 and TRAF3 are recruited to TRIF to activate NF-κB and IRF3 signaling. NAP1 and SINTBAD are required for the activation of TBK1/IKK-i, which phosphorylate IRF3 (Takeuchi and Akira, 2010).

## Inflammasome

The inflammasomes are a group of multiprotein complexes that mediate the activation of inflammatory caspases, especially caspase-1, leading to the maturation of cytokine IL-1 $\beta$  and IL-18 and the occurrence of pyroptosis (Vladimer et al., 2013). The key components of inflammasome complexes are NOD-like receptors (NLRs), which belong to another family of PRRs and play important roles in host defense. The NLRs are a large family of cytosolic sensors, with 23 members in humans and 34 members in mice (Bryant and Fitzgerald, 2009). NLRs share a common tripartite structure, comprised of a C-terminal leucine-rich repeat (LRR) domain, a central NACHT/NOD domain (nucleotide-binding oligomerization domain), and a variable N-terminal domain (Bryant and Fitzgerald, 2009). The LRR domain is probably involved in directly or indirectly recognizing PAMPs or DAMPs. The variable N-terminal domain, which mediates protein-protein interaction, can be either a caspase recruitment and activation domain (CARD), a Pyrin domain (PYD), or a baculovirus inhibitor of apoptosis repeat domain (BIR) (Bryant and Fitzgerald, 2009). The NLR members that constitute inflammasome complexes share an N-terminal PYD domain or an N-terminal CARD domain, and are thereby referred as NLRP or NLRC respectively, including NLRP1, NLRP3, NLRP6, NLRP12, and NLRC4 (IPAF). Another inflammasome complex does not contain any NLRs, but instead contains a HIN200 family protein, AIM2 (Absent in melanoma 2), which consists of a HIN200 and a PYD domain (Bryant and Fitzgerald, 2009; Vladimer et al., 2013).

The inflammasomes can be activated by a wide range of different stimuli, including diverse microbial, environmental, or endogenous danger signals. Upon stimulation, the components of inflammasome start to assemble through oligomerization, and recruit pro-caspase-1 directly via a CARD homotypic interaction (e.g., NLRP1 or NLRC4 inflammasomes) or indirectly by the help of the adaptor ASC (apoptosis-associated speck-like protein containing a CARD), which interacts

with NLRs through PYD domains (e.g., NLRP3 or AIM2 inflammasomes) (Stutz et al., 2009). The exact mechanism underlying inflammasome formation and activation is still not well understood. Once the pro-caspase-1 is recruited to inflammasome complex, these pro-enzymes undergo auto-cleavage induced by the proximity of two or more caspase-1 monomers, leading to the cleavage of the pro-enzyme into 20-kDa (p20) and 10-kDa (p10) subunits (Stutz et al., 2009). Active caspase-1, acting as a heterodimer of p20 and p10 subunits, then proteolytically cleaves pro-IL-1 $\beta$  and pro-IL-18, enabling the release of mature IL-1 $\beta$  and IL-18 cytokines. Mature IL-1 $\beta$  is a potent pyrogen with multiple pleiotropic functions, including promoting the development and differentiation of T, B, and hematopoietic cells, initiating the acute phase responses and participating in cell proliferation and apoptosis. Due to its highly potent pro-inflammatory activities, the synthesis, processing and release of IL-1 $\beta$  are tightly regulated, requiring two separate signals and processes. First, “signal 1”, frequently initiated by different PRRs (like the TLRs), induces NF- $\kappa$ B and MAPK activation, which results in the transcription and production of pro-IL-1 $\beta$ . With pro-IL-1 $\beta$  in the cytosol, other stimuli induce “signal 2” which leads to the processing of these pro-IL-1 $\beta$  into mature cytokines through caspase-1-mediated proteolysis. These two distinct processes ensure the safety of producing and releasing mature IL-1 $\beta$  (Bryant and Fitzgerald, 2009; Vladimer et al., 2013).

Although caspase-1 is defined as the canonical caspase activated by the inflammasomes to mediate IL-1 $\beta$  and IL-18 maturation, recent studies have shown that other non-canonical caspases, including caspase-11 and caspase-8, are also involved. Firstly, Kayagaki *et al.* indicated that caspase-11 is critical for caspase-1 activation and IL-1 $\beta$  production in macrophages infected with *E. coli*, *Citrobacter rodentium* or *Vibrio cholerae* (Kayagaki et al., 2011). More importantly and surprisingly, they also showed that the existing *Caspase 1*<sup>-/-</sup> mice that were routinely being used at that time, also lack caspase-11 as a result of backcrossing a passenger mutation from the

caspase-11-deficient 129 mouse strain into C57BL/6 mice. Thus, these mice were functionally caspase-1/caspase-11 double knockouts (Kayagaki et al., 2011). Through using caspase-1 or caspase-11 single knockout macrophages, they found that caspase-11 triggers pyroptosis independently of caspase-1. However, for IL-1 $\beta$  release, caspase-11 is proposed to cooperate with NLRP3, ASC and caspase-1 to trigger non-canonical inflammasome activation (Kayagaki et al., 2011). Subsequent studies by both Rathinam et al. and Broz et al. demonstrated that TLR4-TRIF activates caspase-11 via type I IFN signaling during infection with Gram-negative bacteria, including enterohemorrhagic *E. coli* (EHEC), *Citrobacter rodentium* and *Salmonella typhimurium* (Broz et al., 2012; Rathinam et al., 2012). Activated caspase-11 synergizes with NLRP3 inflammasome to regulate caspase-1 activation and IL-1 $\beta$  release, and simultaneously leads to caspase-1-independent cell death (Rathinam et al., 2012). These results suggest that caspase-11 orchestrates both caspase-1-dependent and -independent effects, possibly through distinct mechanisms.

Caspase-8 is an important initiator caspase in apoptosis (will be described later in this thesis). However, recent investigations have indicated that caspase-8 can also induce IL-1 $\beta$  and IL-18 cleavage independent of caspase-1 when stimulated with TLR ligands, poly(I:C) and LPS (Maelfait et al., 2008), death-inducing receptor Fas ligand (FasL) (Bossaller et al., 2012), or fungi and mycobacteria sensed by a C-type lectin receptor dectin-1 (Gringhuis et al., 2012). In these studies, caspase-8-mediated IL-1 $\beta$  maturation does not require any known NLRs, although ASC may be required. In addition, caspase-8 deficiency in dendritic cells facilitates LPS-induced activation of the NLRP3 inflammasome and IL-1 $\beta$  secretion. This effect is dependent on RIP1, RIP3, MLKL and PGAM5, all of which are involved in programmed necrosis (Kang et al., 2013), which will be described in more detail later in this thesis. Paradoxically, TLR priming plus IAP (inhibitor of apoptosis) protein inhibition triggers the maturation and secretion of IL-1 $\beta$  that is

mediated not only by the NLRP3-caspase-1 inflammasome, but also by the caspase-8 non-canonical inflammasome. Interestingly, both NLRP3-caspase-1 and caspase-8 inflammasomes require RIP3 and ROS production (Vince et al., 2012). These controversial results suggest that caspase-8 can regulate IL-1 $\beta$  activation by two different mechanisms. Active caspase-8 can directly cleave IL-1 $\beta$  via a caspase-1-independent pathway, while caspase-8 protein can block RIP3-mediated inflammasome activation and IL-1 $\beta$  secretion. Caspase-8 deficiency triggers the disruption of cell homeostasis and leads to a failure in blocking RIP3-mediated activation of the NLRP3 inflammasome (Wen et al., 2013).

## **Host cell death**

Cell death is important to maintain tissue homeostasis in multicellular organisms. When encountering pathogen invasion, host cell death is elicited as an intrinsic innate immune defense mechanism. Cell death plays a critical role in host-pathogen interactions. However, the exact function seems to be context-dependent. On one hand, inducing immune cell death can eliminate the replication niche of intracellular bacteria, thus inhibiting the proliferation of these pathogens and exposing them to bactericidal mechanisms. On the other hand, elimination of key immune cells can diminish the ability of those cells to respond to infection, enable bacteria to evade the host immune defenses, and prompt the dissemination of bacteria in the host. Thus, eliciting host cell death is a double-edged sword with either protective or harmful outcomes in terms of controlling infection (Ashida et al., 2011; Lamkanfi and Dixit, 2010). Apoptosis, necrosis and pyroptosis represent the three major modules of cell death during infection. The mechanisms of the three cell death modules are different and complicated. The choice of death mode is also complex, dependent on different factors, ranging from the nature of the microbial infection, the site of infection and the stage of infection. Pathogens are also equipped with different effectors



and toxins that may target the host cell death machinery and manipulate different signaling pathways leading to host cell death (Ashida et al., 2011; Lamkanfi and Dixit, 2010).

## **Apoptosis**

Apoptosis is the best characterized mode of programmed cell death. The term “Apoptosis” derives from Greek language and means the leaves “falling off” from trees in autumn. It was first used in 1972 by Kerr *et al.* (Kerr et al., 1972). Apoptosis is crucial for embryonic development, tissue homeostasis and other physiological processes, as well as a number of pathological diseases, such as cancer, autoimmune disorders and pathogen infections. Apoptotic cells display distinct morphologies with membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies (Strasser et al., 2000). Normally, apoptotic cell death is triggered by two different pathways: the extrinsic or death receptor pathway and intrinsic or mitochondrial pathway. Both pathways converge on the activation of the cascade of cysteinyl aspartate-specific proteases (caspases) (Thornberry, 1998). Different initiator caspases (caspase-2, 8, 9, or 10) are activated by either extrinsic or intrinsic stimuli, and then the executioner caspases (caspase-3, 6, 7) are directly cleaved and activated by respective initiator caspases. Activated executioner caspases then cleave several substrates, such as poly-ADP ribose polymerase-1 (PARP-1), the inhibitor of caspase-activated DNase (ICAD) and so on, ultimately leading to the occurrence of apoptosis (Strasser et al., 2000; Thornberry, 1998).

The extrinsic pathway is initiated by binding of extracellular ligands to their corresponding transmembrane receptors which are called death receptors. Such ligands belong to the tumor necrosis factor (TNF) superfamily, including TNF $\alpha$ , Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Upon ligands ligation, specific cytoplasmic adaptors, such as

FADD, RIP1 or TRADD, are recruited to the death receptors to form a death-inducing signaling complex (DISC) (Boatright et al., 2003; Daniel, 2000). FADD then recruits monomeric pro-caspase-8 to the DISC complex through death effector domain (DED) interactions. DISC formation subsequently facilitates the dimerization of pro-caspase-8, which results in its activation and auto-proteolytic cleavage (Fuentes-Prior and Salvesen, 2004; van Raam and Salvesen, 2012). Pro-caspase-8 cleaves itself between the DEDs and the large subunit, leading to the release of active (cleaved) caspase-8 from the DISC to directly activate the executioner caspases (Boatright et al., 2003; Strasser et al., 2000). The interaction between FADD and pro-caspase-8 can be regulated by cellular FLICE-like inhibitory protein (cFLIP). All cFLIP isoforms, including long, short and Raji isoforms, contain DED domains which compete with pro-caspase-8 to bind to FADD thus prevent the interaction between FADD and pro-caspase-8. cFLIP long (cFLIP<sub>L</sub>) isoform also contains a caspase-like domain in which the active-center cysteine residue is substituted by a tyrosine residue. cFLIP<sub>L</sub> can form heterodimer with caspase-8 to mediate the activation and partial self-processing of caspase-8 (Irmeler et al., 1997; Yu and Shi, 2008).

The intrinsic apoptosis pathway is induced by different stimuli, including growth-factor depletion, DNA damaging agents like UV irradiation and chemotherapeutics, etc. These stimuli induce mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c*, SMAC/Diablo and HtrA2/Omi into the cytosol from the mitochondrial intermembrane space (Cain et al., 2002; Liu et al., 1996). Released cytochrome *c* interacts with the adaptor Apoptosis protease activation factor-1 (Apaf-1) to form the apoptosome complex, which serves as a scaffold to recruit and activate initiator caspase-9 (Cain et al., 2002).

In addition to its crucial role in various physiological conditions, like embryonic development and maintenance of tissue homeostasis, apoptosis also contributes to regulating host-pathogen

interactions. Many bacterial pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Yersiniae*, can induce host cell apoptosis to destroy the immune protective cells of host and thus facilitate their infection. In contrast, some bacteria, including *Legionella pneumophila*, *A. phagocytophilum*, *Chlamydia* spp. and others, inhibit macrophage apoptosis to maintain their replication niches via different mechanisms (Lamkanfi and Dixit, 2010; Rudel et al., 2010).

## Necrosis

Necrosis was first described as accidental cell death with swelling of organelles, plasma membrane rupture and cell lysis. Recent studies have indicated that certain types of necrosis can also be tightly regulated, and this has given rise to the new term: necroptosis, which means programmed necrosis. The serine-threonine kinase Receptor-interacting protein 1 (RIP1) was the first identified regulator of necrosis. It is required for Fas or TNF $\alpha$ -triggered necrosis in Jurkat human T lymphocyte cells or mouse embryonic fibroblasts (MEFs) respectively (Holler et al., 2000). In 2009, three groups identified RIP3, another RIP family member, as a key regulator of death receptor-induced necrosis (He et al., 2009; Cho et al., 2009; Zhang et al., 2009). The availability of RIP3<sup>-/-</sup> mice facilitates the study of necrosis in pathophysiology and since then, necroptosis and the related molecules have gained intensive investigations. RIP1/RIP3-mediated necroptosis has been found to play an important role in various pathophysiological conditions, such as microbial infections, Crohn's diseases, acute pancreatitis, atherosclerosis and other inflammation-mediated tissue damages (reviewed by Moriwaki and Chan, 2013). Many stimuli can induce necroptosis, including death cytokines of the TNF family, intracellular ATP depletion, mitochondrial depolarization, poly-(ADP-ribose) polymerase (PARP) activation, increased reactive oxygen species (ROS) and several viruses like vaccinia virus, herpes virus, mouse cytomegalovirus (Vanlangenakker et al., 2012). Upon exposure to these stimuli, RIP1 interacts

with RIP3 through their RHIM domains, a hydrophobic patch of  $\beta$  sheet flanked by unstructured coiled-like residues. The interaction between RIP1 and RIP3 leads to the assembly of necrosome, which shows a filamentous structure with classical characteristics of  $\beta$ -amyloids (Li et al., 2012). For the downstream execution of necroptosis, MLKL (mixed-lineage kinase domain-like) and mitochondrial protein phosphatase PGAM5 have been recently identified as RIP3 substrates. Both MLKL and PGAM5 have been indicated as components of necrosome complex and are required for the processing and execution of necrosis (Sun et al., 2012; Wang et al., 2012). MLKL can interact with RIP3 through its C-terminal kinase-like domain, which is phosphorylated by RIP3 at Thr357 and Ser358 residues, and this interaction between MLKL and RIP3 is dependent on the kinase activity of RIP3 (Sun et al., 2012). Knocking down MLKL expression or treating cells with human MLKL inhibitor necrosulfonamide, can arrest the necrosis induced by TNF $\alpha$  + Smac mimetic + z-VAD-fmk (Sun et al., 2012). The role of MLKL in necroptosis is further confirmed by the generation of *Mkl* knockout mice by transcription activator-like effector nucleases (TALENs)-mediated gene disruption (Wu et al., 2013). *Mkl* knockout mice are viable, healthy, fertile and do not show any abnormalities in the development of T cells, macrophages and neutrophils, similar to RIP3 knockout mice. However, mouse embryonic fibroblasts (MEFs) and macrophages from the *Mkl*-deficient mice are resistant to necrosis induced by LPS plus zVAD, or TNF plus Smac mimetic and zVAD. Moreover, *Mkl* knockout mice are protected from cerulean-induced acute pancreatitis, which is recognized as a necrosis-associated disease and requires RIP3 for disease progression (Wu et al., 2013). A distinct *Mkl* deficient mouse generated by another group shows similar phenotypes and results, confirming that MLKL is required for necroptosis induced by TNF $\alpha$  (Murphy et al., 2013). Several very recent studies further clarified the mechanism about how MLKL mediates TNF $\alpha$ -induced necroptosis (Cai et al., 2014; Chen et al., 2014; Wang et al., 2014). Since Thr357 and Ser358 of MLKL are phosphorylated during necroptosis and mutations of these two residues into

alanine suppress necrosis progression (Sun et al., 2012), a monoclonal antibody that specifically recognizes phosphorylated Thr357/Ser358 of MLKL can be utilized as a specific cellular marker to detect necroptosis. Through the development of this monoclonal antibody and using it in a series of biochemical assays, Wang et al. (2014) demonstrate that the phosphorylation of MLKL induced by RIP3 in necrosis triggers MLKL to form an oligomer, which binds to phosphatidylinositol lipids and cardiolipin of membrane compartment. The oligomerization of phosphorylated MLKL enables the necrosome to translocate from the cytosol to the plasma and intracellular membranes, leading to membrane disruption and necrosis occurrence. Moreover, the antibody against phospho-MLKL is able to detect necroptotic hepatocytes in human liver biopsy samples from patients with drug-induced liver injury, a disease that involves necroptosis for the disease progression confirmed by RIP3 knockout mice, but not in healthy donor's liver samples (Wang et al., 2014). The results of two other studies support that the oligomerization of MLKL and the translocation of the MLKL oligomers to plasma membrane are essential for TNF $\alpha$ -induced necrosis (Cai et al., 2014; Chen et al., 2014). All these investigations are carried out utilizing the necrosis model induced by TNF $\alpha$ , Smac mimetic and zVAD-fmk treatment. Thus, the role of MLKL in necrosis needs to be expanded in other models of necrosis stimuli. In addition to MLKL, the mitochondrial protein phosphatase PGAM5 has been identified as another substrate of RIP3. Upon necrosis induction by TNF $\alpha$ , PGAM5 is recruited to the RIP1-RIP3-MLKL necrosome complex and PGAM5 subsequently recruits the mitochondrial fission factor Drp1 and dephosphorylates it at serine 637, thereby activating Drp1's GTPase activity. Activated Drp1 has been proposed to trigger mitochondrial fragmentation, which is observed in both apoptosis and necrosis and is required for necrosis execution (Wang et al., 2012). A subsequent study suggested that Drp1 is not required for RIP3-induced necroptosis, through using stable cell lines of wild-type, *Mkl<sup>-/-</sup>* and *Drp1<sup>-/-</sup>* MEFs that express a 4-hydroxytamoxifen (4HT)-inducible RIP3-gyrase construct. In this study, necroptosis of MEFs is triggered by induced expression and

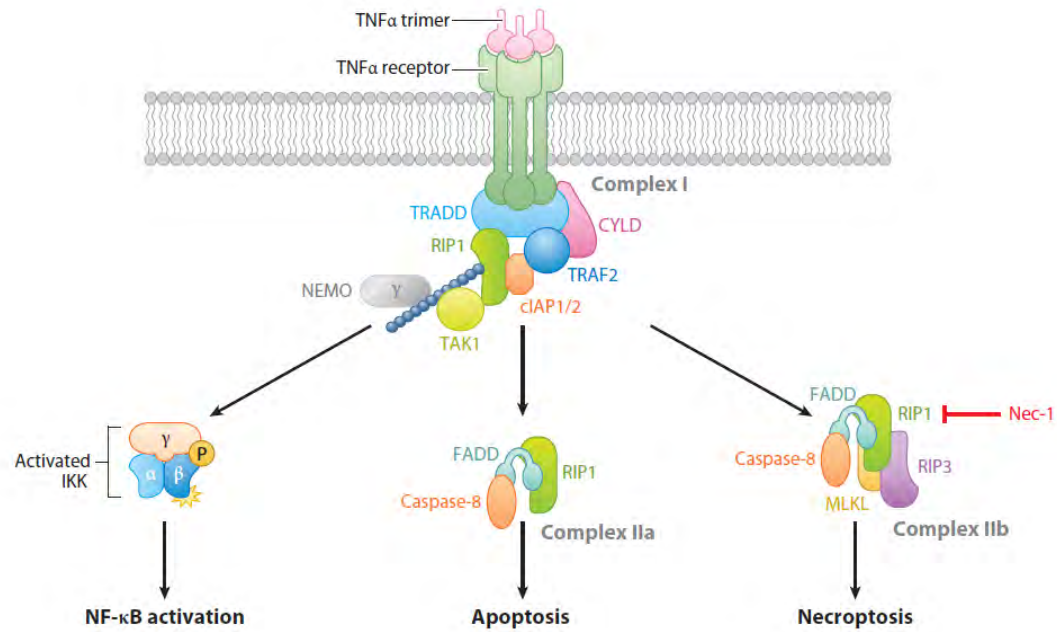
forced dimerization of RIP3, instead of using the combinational treatment of TNF $\alpha$ , Smac mimetic and zVAD-fmk as previously used by Wang et al. (Moujalled et al., 2014; Wang et al., 2012). Hence, different stimuli and different cell lines make the context complicated, leading to different results and conclusions. These findings suggest that there might be different substrates and mechanisms that mediate necrosis execution, in different cell types and by different necrotic stimuli. Further studies in various models are needed to elucidate the necrosis execution pathways.

### **The interplay between RIP1, Caspase-8 and RIP3 in apoptosis, necrosis and inflammation**

The necrosis induced by combined TNF $\alpha$ , Smac mimetic and zVAD-fmk treatment is the most extensively characterized cellular model of necrosis. Much of our knowledge about the mechanism of necroptosis has been achieved by studying this model. However, TNF $\alpha$  can trigger three different outcomes based on cellular status and molecular context: NF- $\kappa$ B and MAPK pathways activation, apoptosis and necroptosis (Christofferson et al., 2014; Festjens et al., 2007). Upon TNF $\alpha$  ligation to its receptor TNFR1, multiple proteins are recruited to TNFR1 at the plasma membrane to form the signaling complex termed as ‘complex I’ that consists of TRADD, RIP1, cIAP1/2, TRAF2 and TRAF5. As E3 ligases, cIAP1/2 mediate the polyubiquitination of RIP1, which provides a docking site to recruit the IKK complex and the TAK1-TAB1-TAB2 complex, leading to the activation of NF- $\kappa$ B and MAPK pathways. Thus, complex I functions as a scaffold to recruit downstream kinases and effector proteins to activate NF- $\kappa$ B and MAPK pathways, leading to cell survival and proliferation (Fig. 1.2) (Christofferson and Yuan, 2010; Festjens et al., 2007). When RIP1 is deubiquitinated by the deubiquitinase CYLD (cyclindromatosis), it translocates from the plasma membrane to cytosol to form a second complex (complex IIa). Complex IIa often (but not invariably) contains RIP1, Caspase-8, FADD

(Fas-associated protein with a death domain), cFLIP and RIP3 (Ofengeim and Yuan, 2013). In complex IIa, caspase-8 is activated and exerts two functions. On one hand, active caspase-8 initiates the caspase cascade, leading to the fate of apoptotic cell death. On the other hand, caspase-8 can also inhibit RIP3-mediated necroptosis, potentially through cleaving RIP1 and RIP3 (Chan et al., 2003; Feng et al., 2007). cFLIP and FADD are also required to block necroptosis (Dillon et al., 2012). Hence, when caspase-8, FADD or cFLIP are blocked either by pharmacological inhibitors or by genetic deficiency, the inhibitory effect on RIP1 and RIP3 is abrogated, leading to the formation of another complex (complex IIb). Complex IIb is also called necrosome, which is composed of RIP1, RIP3 and MLKL. This complex triggers the necrosis induction (Fig. 1.2) (Christofferson et al., 2014; Vandenabeele et al., 2010). Therefore, the cell fate after TNF $\alpha$  stimulation is decided by the interplay between RIP1, Caspase-8 and RIP3.

RIP1 and RIP3 belong to the RIP family. Both of them have an active N-terminal kinase domain, and a RIP homotypic interaction motif (RHIM) at the C terminus of RIP3, but in the intermediate domain of RIP1. RIP1 also has a C-terminal death domain (DD), which mediates the interaction between RIP1 and TRADD, Fas and other DD-containing proteins. The RHIM domain mediates the interaction between RIP1 and RIP3, and is required for induction of necrosis (Moquin and Chan, 2010; Ofengeim and Yuan, 2013). The kinase domain of RIP1 and RIP3 is also critical for necrosis. RIP1 kinase activity is required for the interaction of RIP1 with RIP3, and the formation of necrosome complex. Necrostatin-1 (Nec-1), a small molecule that allosterically blocks the kinase activity of RIP1, can inhibit the induction of necrosis, but leave RIP1-mediated NF- $\kappa$ B and



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**Figure 1.2 Schematic representation of the signaling pathways induced by TNF $\alpha$ .**

Activation of TNFR1 by TNF $\alpha$  leads to three distinct outcomes which are regulated by different complexes: the formation of complex I leads to NF- $\kappa$ B activation; complex IIa leads to apoptosis and complex IIb to necroptosis (Christofferson et al., 2014).



MAPKs activation unaffected (Degterev et al., 2005). Nec-1 treatment abolishes the interaction of RIP1 and RIP3, suggesting that the kinase activity of RIP1 is crucial for assembly of the RIP1-RIP3 necrosome complex (Cho et al., 2009; He et al., 2009).

Caspase-8, as a cysteine protease, is originally recognized as an initiator caspase in extrinsic apoptosis pathway induced by the death receptors of TNF superfamily. Later it has been found to harbor other non-apoptotic functions as well, including mediating the NF- $\kappa$ B activation in T cells and B cells (Kang et al., 2004; Su et al., 2005). The non-apoptotic role of caspase-8 has started to be clarified through various transgenic mouse models. Caspase-8 deficient mice are embryonic lethal, die between embryonic day 10.5 (E10.5) and E11.5, with extensive necrotic cell death and vascular cardiac and hematopoietic defects (Varfolomeev et al., 1998). The additional deficiency of RIP3, however, can rescue the embryonic lethality of *Casp8*<sup>-/-</sup> mice. *Casp8*<sup>-/-</sup>*Rip3*<sup>-/-</sup> double knockout mice are viable and mature into fertile adults with a full immune complement of myeloid and lymphoid cells (Kaiser et al., 2011; Oberst et al., 2011). When getting over the first few months of age, these *Casp8*<sup>-/-</sup>*Rip3*<sup>-/-</sup> mice develop splenomegaly lymphadenopathy, that are due to a marked accumulation of B220<sup>+</sup>, CD3<sup>+</sup> T cells in the secondary lymphoid tissues. This lymphadenopathy phenotype is similar to Fas-deficient (*lpr/lpr*) mice which have abnormal accumulation of B220<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> T lymphocytes in the periphery and develop the autoimmune lymphoproliferative syndromes (ALPS) (Siegel et al., 2000). Through reconstituting *Casp8*<sup>-/-</sup>*Rip3*<sup>-/-</sup> DKO mouse embryonic fibroblasts (MEF) with caspase-8, non-cleavable caspase-8<sup>DA</sup> or catalytically inactive caspase-8<sup>CA</sup>, it was demonstrated that apoptosis mediated by caspase-8 depends on cleavage of caspase-8 which is consistent with previous studies, while the inhibitory effect on RIP3-mediated necrosis by caspase-8 does not require its cleavage, but depends on its

Genotype of mice	Phenotype	References
<i>Caspase-8</i> <sup>-/-</sup>	Embryonic lethal, die between E10.5 and E11.5;	Varfolomeev et al., 1998
<i>Rip1</i> <sup>-/-</sup>	Not embryonic lethal, but die quickly in 1-3 days after birth;	Kelliher et al., 1998
<i>Rip3</i> <sup>-/-</sup>	Healthy and viable as wild type mice;	Newton et al., 2004
<i>Fadd</i> <sup>-/-</sup>	Embryonic lethal during mid-gestation stages	Yeh et al., 1998
<i>cFLIP</i> <sup>-/-</sup>	Embryonic lethal during mid-gestation stages	Yeh et al., 2000
<i>Caspase-8</i> <sup>-/-</sup> <i>Rip3</i> <sup>-/-</sup>	Rescued from embryonic lethality, viable and mature into fertile adults but develop lymphadenopathy when getting old;	Oberst et al., 2011; Kaiser et al., 2011;
<i>Fadd</i> <sup>-/-</sup> <i>Rip1</i> <sup>-/-</sup>	Rescued from embryonic lethality, but die quickly after birth;	Zhang et al., 2011
<i>Fadd</i> <sup>-/-</sup> <i>Rip3</i> <sup>-/-</sup>	Rescued from embryonic lethality, viable and mature into fertile adults but develop lymphadenopathy when getting old;	Dillon et al., 2012
<i>Caspase-8</i> <sup>-/-</sup> <i>Rip1</i> <sup>-/-</sup>	Rescued from embryonic lethality, but die quickly after birth;	Dillon et al., 2014
<i>Rip1</i> <sup>-/-</sup> <i>Rip3</i> <sup>-/-</sup> <i>Casp8</i> <sup>-/-</sup>	Rescued from embryonic lethality, viable and mature into fertile adults but develop lymphadenopathy when getting old;	Dillon et al., 2014; Rickard et al., 2014; Kaiser et al., 2014;
<i>Rip1</i> <sup>-/-</sup> <i>Rip3</i> <sup>-/-</sup> <i>Fadd</i> <sup>-/-</sup>	Rescued from embryonic lethality, viable and mature into fertile adults but develop lymphadenopathy when getting old;	Dillon et al., 2014
<i>Rip3</i> <sup>D161N/D161N</sup>	Embryonic lethal, die around E11.5;	Newton et al., 2014
<i>Casp8</i> <sup>-/-</sup> <i>Rip3</i> <sup>D161N/D161N</sup>	Rescued from embryonic lethality, viable and mature into fertile adults but develop lymphadenopathy when getting old;	Newton et al., 2014
<i>Rip1</i> <sup>D138N/D138N</sup>	Healthy, normal as wild type mice;	Newton et al., 2014
<i>Rip1</i> <sup>K45A</sup>	Healthy, normal as wild type mice;	Berger et al., 2014; Kaiser et al., 2014;
<i>Mlkl</i> <sup>-/-</sup>	Healthy and viable as wild type mice;	Murphy et al., 2013; Wu et al., 2013;

**Table 1.1 The phenotypes of genetically modified mice.**

catalytic activity (Oberst et al., 2011). Oberst et al. also propose that the ability of caspase-8 to inhibit RIP3-mediated necrosis requires the expression of FLIP, and caspase-8-FLIP heterodimers are enzymatically active to inhibit necrosis, which does not require the cleavage of caspase-8 between its large and small subunits. This is consistent with the similar embryonic lethality phenotype of FADD deficient, or FLIP deficient mice to caspase-8 deficient mice (Yeh, 1998; Yeh et al., 2000). These studies suggest that the main non-apoptotic role of caspase-8 is to suppress RIP3-mediated necrosis, potentially through cooperation with FLIP and FADD. This conclusion is partially supported by another work by Zhang et al. (2011). Similar as the embryonic lethality of *Casp8*<sup>-/-</sup> mice, *Fadd*<sup>-/-</sup> mice die during mid-gestation stages with extensive necrotic cell death and elevated expression of RIP1 in embryos (Yeh et al., 1998), whereas RIP1 deficiency restores normal embryonic development of *Fadd*<sup>-/-</sup> mice. Although *Fadd*<sup>-/-</sup> *Rip1*<sup>-/-</sup> double-knockout mice acquire normal embryogenesis, most of them die within 4 days after birth, consistent with *Rip1*<sup>-/-</sup> mice which die 1-3 days after birth (Kelliher et al., 1998). The phenotypes of these genetically deficient mice suggests that FADD can keep RIP1-mediated necrosis in check, probably through acting in concert with caspase-8 to cleave and inactivate RIP1 (Zhang et al., 2011).

The investigations mentioned above suggest that caspase-8 has two different roles in cell death, as inducers of apoptosis and inhibitors of RIP3-mediated necrosis. For RIP1, it can either prompt caspase-8-mediated apoptosis, or coordinate with RIP3 to induce necrosis, depending on its modification and the intracellular circumstance. So far, it looks like that RIP3 only has one role in this game, to induce necrosis. However, the results of a very recent study challenge this conclusion. In contrast to the viability and normality of *Rip3*<sup>-/-</sup> mice, engineered mice expressing catalytically inactive RIP3 D161N (*Ripk3*<sup>D161N/D161N</sup>) die around embryonic day 11.5 (E11.5) with

abnormal yolk sac vasculature (Newton et al., 2014). When the kinase activity of RIP3 is inhibited by mutating Asp<sup>161</sup> of the catalytically important DFG motif to Asn, apoptosis is significantly increased in the *Ripk3*<sup>D161N/D161N</sup> yolk sac vasculature. In addition, the double mutant *Casp8*<sup>-/-</sup> *Ripk3*<sup>D161N/D161N</sup> mice, generated by breeding the *Ripk3*<sup>D161N</sup> allele onto a caspase-8-null background, are viable and show similar lymphadenopathy with *Casp8*<sup>-/-</sup> *Rip3*<sup>-/-</sup> DKO mice, suggesting that RIP3 D161N mutation induce embryonic lethality through prompting caspase-8-dependent apoptosis during embryogenesis (Newton et al., 2014). RIP1 deficiency rescued embryonic lethality caused by RIP3 D161N, but the *Rip1*<sup>-/-</sup> *Ripk3*<sup>D161N/D161N</sup> mice die soon after birth, similar to *Rip1*<sup>-/-</sup> mice, suggesting that although RIP1 deficiency can suppress the apoptosis which leads to embryonic lethality, the other defects caused by RIP1 loss still incite the postnatal lethality and do not require RIP3 kinase activity-dependent necrosis. Newton et al. also constructed the *Ripk3*<sup>D161N/D161N</sup> embryos lacking MLKL, TRIF, DAI, CYLD, TNFR1 or FLIP, and none of them survive beyond E11.5, demonstrating that these proteins are dispensable for RIP3 D161N-induced apoptosis. Last, the *Rip1* kinase dead knock-in mice, with Asp<sup>138</sup> of the HKD motif necessary for RIP1's kinase activity mutated to Asn (*Ripk1*<sup>D138N/D138N</sup> mice), are viable. This is in contrast to the postnatal death of *Rip1*<sup>-/-</sup> mice, suggesting that RIP1 kinase activity is critical for necrosis, but not required for caspase-8-mediated apoptosis. Using the primary cells from *Ripk1*<sup>D138N/D138N</sup> mice, it is further confirmed that RIP1 kinase activity is dispensable for NF-κB and MAPK activation (Newton et al., 2014).

To further investigate the mechanisms of postnatal lethality of *Rip1*<sup>-/-</sup> mice, three very recent studies have constructed *Rip1*<sup>-/-</sup> *Rip3*<sup>-/-</sup> *Casp8*<sup>-/-</sup> or *Rip1*<sup>-/-</sup> *Rip3*<sup>-/-</sup> *Fadd*<sup>-/-</sup> mice, which behave normally after birth in contrast to *Rip1*<sup>-/-</sup> mice, suggesting the postnatal lethality of *Rip1*<sup>-/-</sup> mice is induced by the synergy of caspase-8 and RIP3 (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al.,

2014). Collectively, as summarized in Table 1.1, the phenotypes of various genetically modified mice depict a picture that FADD-caspase-8-FLIP interacts with RIP1-RIP3-MLKL to regulate cell death during embryonic development and postnatal period.

Cell death is closely interlinked with inflammation. During necrosis, cellular components are released and can trigger inflammation. Apoptosis is originally recognized as “silent” cell death, not inducing inflammation. But recent studies also suggest that there is inflammatory responses accompany the induction of apoptotic cell death (Hotchkiss and Nicholson, 2006; Verfaillie et al., 2013). Moreover, caspase-8, RIP1 and RIP3 are found not only contributing to cell death, but also contributing to inflammation. As mentioned earlier in the inflammasome section, caspase-8 can induce the cleavage of pro-IL-1 $\beta$  into mature cytokines, possibly bypassing the canonical known NLR inflammasomes (Maelfait et al., 2008; Gringhuis et al., 2012). RIP3 is also found to have the potential capability to activate NLRP3 inflammasome and IL-1 $\beta$  release, although this capability is blocked by caspase-8 in normal situation. Only when caspase-8 is genetic deficient or chemically inhibited, RIP3-mediated inflammasome activation can be liberated (Kang et al., 2013). Based on these studies, what we can conclude only is that the interplay between RIP1, Caspase-8 and RIP3 during apoptosis, necrosis and inflammation, is complicated and more efforts are needed to clarify these issues.

## **Pyroptosis**

Pyroptosis is a recently defined pro-inflammatory form of cell death which is mediated by inflammasome-induced caspase-1 activation. Pyroptosis shares some morphology characteristics with both apoptosis and necrosis, with nuclear condensation and oligonucleosomal DNA

fragmentation as well as cytoplasm membrane rupture (Bergsbaken et al., 2009). Pyroptosis occurs in response to a number of bacterial infections, including *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Francisella tularensis* and so on (Bergsbaken et al., 2009; Miao et al., 2011). Inflammasome-mediated caspase-1 activation also triggers maturation and release of pro-inflammatory cytokines IL-1 $\beta$  and IL-18, however, studies indicate that pyroptosis is independent of IL-1 $\beta$  and IL-18. Pyroptosis could be either beneficial or detrimental for the host defense against infection, just depending on whether there is mechanism (like neutrophil phagocytosis) to resolve the bacteria released during cell death and tissue inflammation induced by pyroptosis. Without caspase-1 or neutrophil-mediated phagocytosis, the lysis of macrophages and the release of intracellular *Salmonella* during the occurrence of pyroptosis will be detrimental for the host (Broz et al., 2012; Miao et al., 2010).

Although pyroptosis is defined as the cell death induced by caspase-1, recent studies found that non-canonical caspase-11 activation also contributes to caspase-1-independent cell death in response to *E. coli*, *C. rodentium*, *V. cholerae* and *S. Typhimurium* (Broz et al., 2012; Kayagaki et al., 2011). Since the previously used caspase-1 knockout mice also lack a functional allele of Caspase-11, the caspase-1 knockout mice actually turn out to be functional Casp1 Casp11 double knockouts (Kayagaki et al., 2011). Thus, it is worth investigating the individual roles of caspase-1 and caspase-11 in the cell death using real single knockout animals. The study by Broz et al. suggested that wild-type *Salmonella* induces cell death through two separate pathways, canonical pyroptosis (flagellin recognition by NLRC4 which activates caspase-1) and non-canonical pathway which requires caspase-11 (Broz et al., 2012). The cell death mediated by caspase-11 shows similar characteristics as pyroptosis. Regarding the mechanism underlying caspase-11

activation, both Broz et al. and the simultaneous study by Rathinam et al. indicated that TRIF-dependent interferon production is crucial for caspase-11 activation (Rathinam et al., 2012). However, further investigations are needed to clarify the mechanism of caspase-11-induced cell death, and the individual roles of caspase-1 and caspase-11 in pyroptosis.

## ***Yersinia pestis***

### **Overview**

*Yersinia pestis*, a Gram-negative bacteria, belongs to the genus *Yersinia*, which contains three species known to be pathogenic to humans: *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. *Y. enterocolitica* and *Y. pseudotuberculosis* cause food-borne and waterborne enteric diseases. *Yersinia pestis* is the causative agent of plague, which has caused three major pandemics in history, including the infamous Black Death in medieval Europe. The three pandemics have caused the deaths of an estimated 200 million people and had huge impact on western society and human civilization (Perry and Fetherston, 1997). Nowadays, although infection with *Yersinia pestis* can often be treated by timely use of antibiotics and plague outbreaks are rare worldwide, this bacterium is still a concern for bioterrorism and recognized as a category A select agent (Butler, 2009).

*Y. pestis* can be spread via flea bites, which result in classical bubonic plague with the characteristic swollen lymph nodes, or buboes. Septicemic plague occurs when the bacteria enters the blood stream and spreads systemically. Once the infection has become systemic, the bacterium can colonize the lung and can aerosolize when the patient coughs. Hence it can be

spread person-to-person through aerosol droplets, which leads to occurrence of pneumonic plague. The bubonic and septicemic plague are fatal in 30-70% of cases within 5-10 days if not treated with antibiotics, while pneumonic plague has a much shorter incubation period of only 1-3 days and causes a 100% fatality without treatment (Perry and Fetherston, 1997).

*Y. pestis* is a bacterium with high virulence, leading to such serious diseases in a relatively short time. This is achieved by establishing infection and replicating quickly in host tissues, which requires that *Y. pestis* is capable of evading the host innate immune surveillance at the beginning of infection. In order to shut down host's innate immune responses, *Y. pestis* is evolved to possess several mechanisms as its crucial virulence factors, including type III secretion system (T3SS), outer membrane protease Pla with plasminogen activator activity, non-stimulatory LPS synthesis at mammalian temperature 37°C, F1 capsular antigen (Caf1) expressed at higher-temperature (37°C) environment and multiple iron acquisition systems (Cornelis, 2002; Sodeinde et al., 1992). These mechanisms enable *Y. pestis* to invade the host innate immune system very efficiently, and thus make *Y. pestis* a good model infectious agent to study the relationship between host innate system and bacterial infection.

### **Non-stimulatory LPS synthesis at 37°C**

Many Gram-negative bacteria produce a hexa-acylated lipid A and LPS, as one of their important PAMPs, to trigger strong innate immune responses through TLR4-MD2 signaling (Munford, 2008; Rebeil et al., 2004). However, *Y. pestis* synthesizes different lipid A and LPS with varied structures at different growth conditions. At 26°C (flea temperature), the lipid A portion of LPS is mainly hexa-acylated, similarly to the LPS of many other Gram-negative bacteria. When



temperature is shifted to 37°C (mammalian host temperature), the lipid A produced by *Y. pestis* also changes to be tetra-acylated (Kawahara et al., 2002; Knirel et al., 2005; Rebeil et al., 2004). Tetra-acylated lipid A/LPS is antagonistic to human TLR4/MD2 and can poorly activate TLR4-mediated immune pathways, and it is also a weak agonist towards mouse cells (Kawahara et al., 2002; Knirel et al., 2005; Montminy et al., 2006). This lipid A variance phenotype is due to the absence of LpxL, a late-acyl transferase that is responsible for adding a secondary acyl chain on to the tetra-acylated lipid A precursor molecule in *Y. pestis*. At lower temperatures, *Y. pestis* expresses a late acyltransferases LpxP, which is a cold-shock-induced enzyme and supposedly not active at high temperature 37°C. LpxP cooperates with LpxM, another late acyltransferase, to produce the hexa-acylated lipid A/LPS at 26°C (Rebeil et al., 2006). Through expressing *E. coli* or *Y. pseudotuberculosis* LpxL in *Y. pestis*, these modified *Y. pestis* (*Y. pestis-EcLpxL* or *Y. pestis-YtbLpxL*) strains can produce hexa-acylated lipid A/LPS at 37°C and thus can activate TLR4/MD2 pathway to boost a strong innate immune response (Montminy et al., 2006; Vladimer et al., 2012). *In vitro*, *Y. pestis-EcLpxL* or *Y. pestis-YtbLpxL* activate macrophages to produce higher pro-inflammatory cytokines, IL-6 and TNF $\alpha$  than wild type *Y. pestis*. Further *in vivo* investigations by our group have confirmed that the expression of LpxL in *Y. pestis* significantly reduces the virulence in wild-type mice, but not in TLR4 KO mice, suggesting that synthesis of non-stimulatory LPS at 37°C is a virulence strategy utilized by *Y. pestis* to evade the host innate immune responses (Montminy et al., 2006; Vladimer et al., 2012).

### **Type three secretion system (T3SS) and YopJ**

Type three secretion system (T3SS), which is encoded by the virulence plasmid pCD1 and required for virulence of all three pathogenic *Yersinia* species, is a needle-like machinery to inject

several effector proteins into cytoplasm of host cells. These effector proteins are Yops (*Yersinia* outer proteins) and LcrV. There are seven Yop effector proteins, YopE, YopH, YopT, YopM, YpkA (YopO), YopK and YopJ. The expression and activation of T3SS and Yops are temperature regulated. Upon shift of temperature from 26°C (flea temperature) to 37°C, the expression of *Yersinia* T3SS is upregulated (Cornelis, 2002; Viboud and Bliska, 2005). When the bacteria come into contact with the host cells, the T3SS is further activated and the expressed Yop effectors are secreted and delivered into the cytoplasm of host cells (Pettersson et al., 1996). These Yop effectors can modulate host cells signaling pathways and thus interfere with the immune responses of these targeted host cells, attenuating both innate and adaptive immune responses. YopB and YopD are two structure Yops to form the T3SS needle with LcrV. YopE, YopH, YopT and YpkA inhibit dynamics of the actin cytoskeleton and therefore interfere with the phagocytosis of macrophages and neutrophils (Perry and Fetherston, 1997; Viboud and Bliska, 2005). YopE is a GTPase-activating protein and it can inactivate the Rho family of small GTPases. YopH is a protein tyrosine phosphatase. YopT is a cysteine protease to cleave the Rho GTPases and detach the Rho GTPases from the membrane and prevent membrane anchoring. YpkA is an autophosphorylating serine/threonine kinase and can interact with members of the Rho family GTPases. Since Rho family GTPases are critically involved in actin cytoskeleton function, these four Yops target these GTPases to interfere with cytoskeleton and limit the internalization and phagocytosis of *Yersinia* by macrophages (Trosky et al., 2008; Viboud and Bliska, 2005).

YopJ (YopP in *Y. enterocolitica*) is an interesting effector protein with multiple different effects. It was first found to inhibit NF- $\kappa$ B and MAPK pathways and thereby repress the production of pro-inflammatory cytokines, including IL-6 and TNF $\alpha$  (Palmer et al., 1998; Schesser et al., 1998).

Meantime, two groups reported that YopJ can also induce robust apoptotic cell death in macrophages (Mills et al., 1997; Monack et al., 1997). YopJ has a catalytic domain which is similar to cysteine proteases. Alignment of the amino acid sequences revealed a conserved catalytic triad His109-Glu128-Cys172 for cysteine protease in YopJ (Orth et al., 2000). Mutation of the catalytic triad of YopJ abolishes its ability to inhibit NF- $\kappa$ B and MAPK pathways as well as prompt apoptosis (Orth et al., 2000). The molecular mechanism by which YopJ inhibits NF- $\kappa$ B and MAPK pathways has gained intensive investigation. It was first proposed that YopJ functions as a de-SUMOylating protease, but the targets were not identified (Orth et al., 2000). Later studies suggested YopJ acts as a de-ubiquitinase to cleave polyubiquitin chains from TRAF6, which is an essential NF- $\kappa$ B pathway protein (Sweet et al., 2007; Zhou et al., 2005). Until 2006, two groups showed solid biochemical evidence which indicated that YopJ is a serine/threonine acetyltransferase. Using a cell-free signaling system and mass spectrometry (MS/MS) approach, Mukherjee et al. indicated that YopJ can acetylate the critical serine (ser207) and threonine (thr211) residues in the activation loop of MAPKK6 and thereby inhibit MAPKK6's phosphorylation and activation (Mukherjee et al., 2006). Mittal et al. expressed YopJ or YopJ C172A mutant in Hela cells and discovered that YopJ can acetylate the serine and threonine residues of MEK2, IKK $\alpha$  and IKK $\beta$  to inhibit their phosphorylation and activation (Mittal et al., 2006). A subsequent study by Paquette et al. reported that YopJ inhibits *Drosophila*'s innate immune NF- $\kappa$ B pathway (IMD) but not the Toll pathway. YopJ mediates the serine/threonine acetylation and inhibition of TAK1 in both *Drosophila* and mammalian cells, suggesting that TAK1 is another direct target acetylated and inhibited by YopJ (Meinzer et al., 2012; Paquette et al., 2012). TAK1 lies upstream of IKK $\alpha/\beta$ , MKK6 and MKK4 in NF- $\kappa$ B and MAPK pathways and this suggests that the acetyltransferase activity of YopJ is not limited to MAP2 and IKK $\alpha/\beta$  kinases, and YopJ may have more other targets in host cells.

These above studies elegantly explain how YopJ targets and inhibits NF- $\kappa$ B and MAPK signaling pathways. For the underlying mechanism of YopJ-induced apoptosis, it was believed that the NF- $\kappa$ B and MAPK pathways inhibitory activity of YopJ suppresses the transcription and expression of pro-survival genes and anti-apoptotic genes, leading to the apoptotic death in macrophages. However, through expressing two inhibitors of the NF- $\kappa$ B pathway, I $\kappa$ B $\alpha$  superrepressor or A20 in macrophages, Zhang et al. indicated that inhibition of the NF- $\kappa$ B pathway is not sufficient for rapid *Yersinia*-induced apoptosis. Even when macrophages expressing A20 were pretreated with combined three MAPK pathways' inhibitors, the apoptosis level induced by YopJC172A mutant is still lower than wild type *Yersinia*, suggesting that there is other mechanism targeted by YopJ to trigger apoptotic cell death (Zhang et al., 2005). During *Yersinia* infection, TLR4 plays an important role in mediating NF- $\kappa$ B and MAPK pathways activation by LPS. When NF- $\kappa$ B pathway or protein synthesis is inhibited, TLR4 activation will prompt cell death (Ruckdeschel et al., 1998, 2001). Since *Yersinia* produces both LPS and NF- $\kappa$ B inhibitor, YopJ, it is proposed that YopJ potentiates the apoptotic signaling of LPS mediated by TLR4 through inhibiting NF- $\kappa$ B pathway (Ruckdeschel et al., 2001). Results from both the Ruckdeschel and Bliska groups indicated that although macrophages deficient in TLR4 function are more resistant to *Yersinia*-induced cell death, there are still around 50% of apoptosis remaining in TLR4 deficient cells, suggesting that TLR4 contributes to YopJ-mediated apoptosis but is not absolutely required (Haase et al., 2003; Zhang and Bliska, 2003). Interestingly, regarding the two adaptors downstream of TLR, TRIF deficient cells show similar partial protection from *Yersinia*-induced cell death as TLR4 deficient cells. In contrast, MyD88 knockout macrophages are not protected at all and with similar cell death rate as wild type cells (Ruckdeschel et al., 2004). Moreover, in dendritic cells, YopP of *Y. enterocolitica* induces caspase cleavage but also triggers a caspase-

independent necrosis-like cell death (Gröbner et al., 2006). The same group subsequently demonstrated that *Y. enterocolitica* infection induces the formation of a FADD/caspase-8/RIP1 DISC complex and caspase-8, caspase-3 activation in dendritic cells (Gröbner et al., 2007). As we mentioned in last section of cell death pathways, TRIF is able to interact with RIP1 via their RHIM domains, leading to programmed necrosis through RIP1 and RIP3. These studies suggest that *Yersinia* YopJ/YopP-mediated cell death might involve TLR4, TRIF, RIP1 and caspase-8, but how these molecules contribute to the process still remain elusive.

Beyond the activity to inhibit NF- $\kappa$ B and MAPK pathways and induce apoptosis, YopJ has been found to induce inflammasome activation, leading to caspase-1 cleavage, IL-1 $\beta$  and IL-18's maturation and release (Lilo et al., 2008; Vladimer et al., 2012), although several mechanisms may be involved and are still under investigation. Zheng et al. suggested that YopJ does not directly activate the NLRs, but its inhibitory activity on the NF- $\kappa$ B pathway leads to NLRP3 inflammasome activation (Zheng et al., 2011). Our group also observed that both NLRP12 and NLRP3 are inflammasome components which are required for optimal *Y. pestis*-induced caspase-1 activation and IL-1 $\beta$ , IL-18 secretion. The production of IL-1 $\beta$  and IL-18 are markedly reduced, but not completely abrogated, in NLRP12 KO and NLRP3 KO compared to wild type BMDMs, suggesting there is redundancy or cooperation between NLRs to mediate inflammasome activation by *Y. pestis*. Moreover, NLRP12 KO, NLRP3 KO, IL-1 $\beta$  KO, IL-1R1 KO and IL-18 KO mice are more susceptible to *Y. pestis*-ytlpL infection, suggesting the inflammasome pathways contribute to innate response thus protective effect against *Yersinia* infection (Vladimer et al., 2012). In addition, as other pore-forming toxins or specialized secretion systems of bacterial pathogens, T3SS machinery itself can also induce both NLRP3 and NLRC4 inflammsomes' activation (Brodsky et al., 2010). Therefore, there are at least two independent

mechanisms utilized by *Yersinia* to induce inflammasome activation and caspase-1 cleavage. However, under normal conditions, another *Yersinia* T3SS secreted effector protein, YopK, can interact with the T3SS translocon, regulate effector proteins translocation and prevent the detection of the T3SS by host cell, and thus block the T3SS-mediated inflammasome activation. Hence, the inflammasome-mediated caspase-1 activation is mainly due to YopJ in wild type *Yersinia* infection. When YopK is deficient, the T3SS-induced inflammasome assembly will stand out to induce rapid caspase-1 cleavage and IL-1 $\beta$ , IL-18 secretion (Brodsky et al., 2010). This also explains why caspase-1 activation is inhibited when infected with *Yersinia*  $\Delta$ YopJ mutant which has an intact functional bacterial T3SS. A more recent study indicated that YopM, another Yop effector of T3SS, can directly bind to caspase-1 to arrest inflammasome assembly and activation, although this was only implicated with *Y. pseudotuberculosis* and the role of YopM in *Y. pestis* is unknown (LaRock and Cookson, 2012). For *Yersinia*-induced NLR inflammasomes activation, including NLRP3, NLRC4 and NLRP12, it seems like that different Yop effectors, YopJ, YopK, YopM as well as T3SS translocon itself are involved, but more studies are need to elucidate the tangled relationship between these molecules and their respective roles.

It is impressive that this single effector protein, YopJ, exerts so many distinct effects *in vitro*, inhibiting NF- $\kappa$ B and MAPK signaling pathways, inducing rapid apoptosis of macrophages and dendritic cells, as well as activating inflammasomes and caspase-1 cleavage, resulting in IL-1 $\beta$  and IL-18 maturation and secretion. During these effects, NF- $\kappa$ B inhibition by YopJ will repress the “signal 1” of mature IL-1 $\beta$  secretion, dampening the transcription and expression of pro-IL-1 $\beta$ . But at the same time, YopJ can prompt “signal 2”, and trigger the caspase-1 cleavage to process pro-IL-1 $\beta$  into mature cytokines. Therefore, for mature IL-1 $\beta$  production, YopJ itself shows

contradictory and opposing effects. With such impressive effects *in vitro*, it is interesting to investigate the role of YopJ in *Yersinia* virulence through *in vivo* infections. Results indicate that the role of YopJ/YopP in virulence varies in different *Yersinia* species and in different inoculation models. In *Y. pseudotuberculosis*-induced gastrointestinal disease via the oral infection route, YopJ has been shown to induce apoptosis in infected mesenteric lymph nodes or spleens and this correlates with increased bacterial replication in the tissues. Moreover, the oral LD<sub>50</sub> for a *yopJ* mutant *Y. pseudotuberculosis* increases 64-fold compared with wild type bacteria in BALB/c mice, and the *yopJ* mutant strain is dampened for systemic spread from the Peyer's patches to other lymphoid tissues (Monack et al., 1998). Through a sublethal intragastric mouse infection model by *Y. pseudotuberculosis*, YopJ is demonstrated to promote sustained systemic colonization in C57BL/6 mice, which is associated with the cytotoxicity of YopJ. Moreover, wild type *Y. pseudotuberculosis* induces higher levels of IL-18 and IFN- $\gamma$  cytokines than the *yopJ* mutant strain in mouse serum, and this correlates with the YopJ-mediated inflammasome-caspase-1 activation and IL-1 $\beta$ , IL-18 maturation effect *in vitro* (Zhang and Bliska, 2010). However, in this infection model, the survival curves for mice infected with three different doses of *yopJ* mutant strain are not significantly different from those mice infected with the wild type *Y. pseudotuberculosis*, suggesting that YopJ is not a critical virulence factor of *Y. pseudotuberculosis* serogroup O:1 strain 32777 (previously known as IP2777) using this specific infection procedure (Zhang and Bliska, 2010). Subsequently, Meinzer et al. (2012) demonstrated that *Y. pseudotuberculosis* YopJ contributes to intestinal barrier dysfunction and intestinal permeability, thus promotes the bacterial dissemination from the gut to mesenteric lymph nodes and spleen. YopJ-induced intestinal barrier dysfunction is suggested to associate with YopJ's inhibitory act on Nod2/RICK/TAK1 pathway, resulting in caspase-1 activation and IL-1 $\beta$  secretion in Peyer's patches. For mouse infection induced by orogastric inoculation, YopJ

deficiency delays the mortality of infected mice significantly, although it does not greatly alter the power of *Y. pseudotuberculosis* to kill mice (Meinzer et al., 2012). At the same time, another group published similar results that *Y. pseudotuberculosis* triggers Peyer's patches barrier dysfunction through inducing caspase-1 activation and IL-1 $\beta$  production (Jung et al., 2012). These studies consistently indicate that YopJ of *Y. pseudotuberculosis* has only a limited impact on global bacterial virulence, by prompting bacterial dissemination via dysfunctional intestinal barrier and this correlates with YopJ-induced apoptotic cell death, caspase-1 activation and IL-1 $\beta$  secretion.

However, for the role of YopJ in *Y. pestis* virulence, the case seems to be different. In septicemic plague model through intravenous infection with an attenuated *Y. pestis* strain, *yopJ* mutant only increases the LD<sub>50</sub> by 1.2- to 1.5-fold compared with wild type bacteria (Straley and Bowmer, 1986; Straley and Cibull, 1989). In a rat model of bubonic plague through intradermal (ID) inoculation with the fully virulent *Y. pestis* 195/P strain,  $\Delta yopJ$  mutant is fully virulent as wild type strain, with similar survival rate, clinical signs, systemic spread and colonization (Lemaître et al., 2006). Additionally, in this infection model, YopJ contributes to more apoptotic cells in the bubo lymph node and suppression of TNF $\alpha$  production in serum, that are consistent with YopJ's *in vitro* effect. However, neither the decreased apoptosis in the bubo nor the increased serum TNF $\alpha$  level by the  $\Delta yopJ$  mutant was sufficient to significantly affect *Y. pestis* virulence (Lemaître et al., 2006).

In summary, through oral infection, YopJ/P of *Y. pseudotuberculosis* and *Y. enterocolitica* contributes to systemic spread of bacteria and barrier dysfunction. But for bubonic plague induced by s.c. infection, YopJ is dispensable and there is no significant phenotype when



infecting with *Y. pestis*  $\Delta$ YopJ. Hence, the *in vivo* virulence role of YopJ might depend on different infection routes and models. The molecular mechanism of YopJ-induced apoptosis and inflammasome activation, and the *in vivo* role of YopJ in different infection models and the underlying mechanism between bacteria-host interaction need further investigation.

## Preface to CHAPTER II

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*Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death.* Proc Natl Acad Sci USA. 2014 May 20;111(20):7391-6.

This chapter represents a thesis project of Dan Weng, who generated all the data except that indicated below. Dan Weng, Egil Lien and Robyn Marty-Roix prepared the manuscript.

Co-authors contributed the following data:

Dan Weng, Robyn Marty-Roix and Egil Lien performed the experiments for Figure 2.18, 2.19 and 2.20;

Reagents, cells and mice: Sandhya Ganesan immortalized the  $RIP3^{-/-}Casp8^{-/-}$  macrophages; Megan K. Proulx and Jon Goguen created the KIM5 $\Delta YopJ$  and *Y. pseudotuberculosis*  $\Delta YopJ$  used for *in vitro* stimulations; Kimberly Pouliot and Jon Goguen created the KIM5 $\Delta caf$  strain; William Kaiser and Edward Mocarski generated the  $RIP3^{-/-}caspase-8^{-/-}$  mice; Michelle Kelliher and Kate Fitzgerald provided  $RIP1^{-/-}$  fetal liver macrophages; Francis K.-M. Chan provided  $RIP3^{-/-}$  mice; Peter Gough, John Bertin and Phillip Harris provided  $RIP3$  inhibitor GSK'872 and novel  $RIP1$  inhibitor GSK'963; Dmitry Shayakhmetov provided the caspase-8 CKO (Caspase-8 fl/fl LysM cre<sup>+/+</sup>) bone marrow cells.

## **CHAPTER II**

### **Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death**

## Abstract

A number of pathogens cause host cell death upon infection, and *Yersinia pestis*, infamous for its role in large pandemics such as the “Black Death” in medieval Europe, induces considerable cytotoxicity. The rapid killing of macrophages induced by *Y. pestis*, dependent upon type III secretion system effector YopJ, is minimally affected by the absence of caspase-1, caspase-11, Fas ligand or TNF $\alpha$ . Caspase-8 is known to mediate apoptotic cell death in response to infection with several viruses and to regulate programmed necrosis (necroptosis), but its role in bacterially-induced cell death is poorly understood. Here we provide genetic evidence for a Receptor-Interacting protein (RIP) kinase-caspase-8-dependent macrophage apoptotic death pathway following infection with *Y. pestis*, influenced by TLR4-TRIF. Interestingly, macrophages lacking either RIP1, or caspase-8 and RIP3, also had reduced infection-induced production of IL-1 $\beta$ , IL-18, TNF $\alpha$  and IL-6; impaired activation of the transcription factor NF- $\kappa$ B; and greatly compromised caspase-1 processing. Cleavage of the pro-form of caspase-1 is associated with triggering inflammasome activity which leads to the maturation of IL-1 $\beta$  and IL-18, cytokines important to host responses against *Y. pestis* and many other infectious agents. Our results identify a RIP1–caspase-8/RIP3-dependent caspase-1 activation pathway following *Y. pestis* challenge. Mice defective in caspase-8 and RIP3 were also highly susceptible to infection and displayed reduced pro-inflammatory cytokines and myeloid cell death. We propose that caspase-8 and the RIP kinases are key regulators of macrophage cell death, NF- $\kappa$ B and inflammasome activation, and host resistance after *Y. pestis* infection.

## Introduction

*Yersinia pestis*, the causative agent of plague, is well known to cause significant cell death upon infection through its type three secretion system (T3SS) effector YopJ (Lilo et al., 2008; Lukaszewski et al., 2005; Philip and Brodsky, 2012). Like the activation of inflammatory pathways to produce cytokines, triggering cell death pathways is a common response of the mammalian immune system to infection. Death of immune cells can eliminate the replication niche of pathogens found within those cells, thus inhibiting the proliferation of the pathogens and exposing them to bactericidal mechanisms (Miao et al., 2010). Conversely, elimination of key immune cells can diminish the ability of those cells to respond to infection. Multiple host and microbial factors control cell death pathways (Lamkanfi and Dixit, 2010). Apoptosis, receptor interacting protein-1 (RIP1) and RIP3-dependent necroptosis, and caspase-1/caspase-11-dependent pyroptosis constitute three major modes of regulated cell death during infection (Lamkanfi and Dixit, 2010; Vanlangenakker et al., 2012). Apoptosis is the best characterized programmed cell death and depends on the caspase family. Various stimuli induce the activation of initiator caspases, including caspase-8, caspase-9 and caspase-10, which subsequently activate the effector caspases, such as caspase-3 and caspase-7.

Several viruses appear to induce caspase-8 dependent apoptosis (Mocarski et al., 2012). Caspase-8 has also been suggested to have additional non-apoptotic functions, such as inhibiting necroptosis (Kaiser et al., 2011; Mocarski et al., 2012; Oberst et al., 2011) and modulation of NF- $\kappa$ B activation in T and B cells (Su et al., 2005). Signaling to the transcription factor NF- $\kappa$ B controls the transcription of cytokines such as IL-6, TNF, pro-IL-1 $\beta$  and pro-IL-18, and stimulates cell survival. *Y. pestis* can induce cell death in macrophages and dendritic cells via the

T3SS effector YopJ (YopP in *Y. enterocolitica*), although it is unclear whether this is entirely by apoptosis (Gröbner et al., 2007; Zheng et al., 2012). All human-pathogenic *Yersiniae* (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) harbor cytotoxic properties towards host cells, and YopJ production is associated with cell death both *in vivo* and *in vitro* (Brodsky and Medzhitov, 2008; Brodsky et al., 2010; Monack et al., 1998; Zhang and Bliska, 2010). YopJ-mediated inhibition of NF- $\kappa$ B by acetylation of IKK $\beta$ , MAP kinase kinases, and TAK1 may modulate macrophage death via effects on inflammatory and pro-survival signals (Greten et al., 2007; Meinzer et al., 2012; Mittal et al., 2006; Mukherjee et al., 2006; Paquette et al., 2012; Philip and Brodsky, 2012). Inflammasome activation, culminating in the activation and processing of caspase-1, leads to the production of IL-18 and IL-1 $\beta$ , key inflammatory cytokines and anti-bacterial defenses, but can also be associated with caspase-1 dependent pyroptotic cell death (Vladimer et al., 2013). YopJ also participates in inflammasome activation (Brodsky et al., 2010; Zheng et al., 2011) leading to a host immune response. Thus, this single bacterial effector may induce both protective and harmful effects for the host. In the present study, we investigated the mechanisms for *Y. pestis*-induced cell death, NF- $\kappa$ B activation and inflammasome activation.

## Materials and Methods

### Mice

Mice were maintained in specific-pathogen-free conditions at UMass Medical School and all experiments involving animals were approved by the Institutional Animal Care and Use Committee of the UMass Medical School. RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> double-deficient mice (Rip3<sup>-/-</sup> xCasp8<sup>-/-</sup>) (DKO) (Kaiser et al., 2011) mice were generated by W. Kaiser and E. Mocarski and were bred 6-10 generations into C57Bl/6. Rip3 (RIP3<sup>-/-</sup>) mice were generated by V. Dixit

(Genentech) (Newton et al., 2004). Caspase-8fl/fl LysM cre<sup>+/+</sup> (CKO) mice on a C57Bl/6 background were generated at the University of Washington with a Caspase-8fl/+ allele provided by S. Hedrick (Beisner et al., 2005). C57Bl/6 mice were bred in-house or from the Jackson Laboratory.

Mice lacking IFN $\alpha$  $\beta$ R1 (Ifnar1<sup>-/-</sup>) were from J. Sprent (The Scripps Research Institute). FasL<sup>-/-</sup> (Fasl<sup>-/-</sup>) and TNF- $\alpha$ <sup>-/-</sup> (Tnf<sup>-/-</sup> on a LCMV CTL P14 background, with corresponding WT controls) mice were from A. Marshak-Rothstein, R. Welsh and The Jackson Laboratories. Mice lacking caspase-1 (Casp1<sup>-/-</sup> ; also containing a caspase-11 mutation), NLRP3 (Nlrp3<sup>-/-</sup>), NLRP3/NLRC4 (Nlrp3<sup>-/-</sup>xIpaf<sup>-/-</sup>), NLRP12 (Nlrp12<sup>-/-</sup>), TLR4 (Tlr4<sup>-/-</sup>), TRIF (Ticam1<sup>-/-</sup>), or MyD88 (Myd88<sup>-/-</sup>) were from S. Akira, M. Starnback or Millennium Inc. as previously reported (Montminy et al., 2006; Vladimer et al., 2012).

Bone marrow transplantation (BMT) was performed on lethally (900 rads) irradiated C57Bl/6 mice that were reconstituted with 4x10<sup>6</sup> cells from the indicated genotypes of mice. Mice were used for infections 6-10 weeks post transplantation, or when 6-12 weeks old (age/sex matched, similar numbers of each sex were used). Mice were infected subcutaneously (s.c) in the nape of the neck or intravenously (i.v.) with 500 CFU of KIM1001-pEcLpxL as reported (Montminy et al., 2006) and monitored for survival twice a day for up to 30 days; or sacrificed at ~42 hours post-infection for cytokine, CFU, tissue histology and cell death analysis. Sample collection was performed as previously described (Vladimer et al., 2012). Spleens from mice infected s.c. with 300 CFU of KIM1001 were harvested 68 hrs post infection. Cytokine amounts from the spleen were normalized by spleen weight to account for any increases in tissue size during active



infection, since RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> mice show signs of markedly enlarged spleens by 6-8 weeks of age due to an increase in T cell numbers (Kaiser et al., 2011).

### Reagents

Necrostatin-1 (Nec-1) was purchased from Enzo Life Sciences. Nigericin was from Sigma-Aldrich. z-VAD-fmk was from Promega. Antibodies were purchased from the following vendors: RIP1 and  $\beta$ -actin (BD Pharmingen); Caspase-8 and cIAP1 (Enzo); Caspase-3, I $\kappa$ B $\alpha$ , pI $\kappa$ B $\alpha$ , pIKK $\alpha$ / $\beta$ , IKK $\alpha$  and p65 (Cell Signaling); Caspase-1 (eBioscience); IL-1 $\beta$  (R&D Systems), RIP3 (ProSci), ASC (Santa Cruz), Histone H3 (Abcam). Pam3Cys was obtained from Invivogen.

### Bacterial Strains and Growth Conditions

*Y. pestis* KIM5 (containing the complete T3SS) or KIM5 $\Delta$ YopJ was grown in tryptose-beef extract (TB) broth with 2.5 mM CaCl<sub>2</sub> overnight with shaking at 26°C. The next day, the bacteria was diluted 1:8 into fresh media, cultured for one hour at 26°C and shifted to 37°C for two hours, or grown continuously at 37°C when indicated. KIM5-EcLpxL was grown similarly, but the culture medium was supplemented with 100  $\mu$ g/ml ampicillin. *Y. pseudotuberculosis* IP2666 and *Y. enterocolitica* 8081 (Vladimer et al., 2012) were grown at 37°C in TB overnight, diluted and further cultured for three hours at 37°C. *S. enterica* serovar *typhimurium* strain SL1344 was provided by M. O’Riordan, and grown at 37°C with shaking. Generation of *Y. pseudotuberculosis* IP2666 $\Delta$ YopJ: an in-frame deletion removing codons 4-287 was created via allelic exchange. PCR products made with primer sets

A (ATAGAGCTCCACTACTGATTCAACTTGGACG)

and B (TCCGATCATTTATTTATCCTTATTCA),

and C (TGAATAAGGATAAATAAATGATCGGATAATGTATTTTGGAAATCTTGCT) and D (GGGTCTAGACTGATGTCGTTTATTTCTGGGTAT), respectfully, were used to make a fused product by overlap PCR using primers A and D (Horton et al., 1989). Generation of *Y. pestis* KIM5  $\Delta$ CaF1 (F1): an in-frame deletion removing codons 11-164 was created via allelic exchange. PCR products made with primer sets

A (ATCGATGAGCTCCGTCACAGTAAGAGCACAACTT),

B (GCAGCAGCAGCAGCATGGCGATAACGGAACTGAT)

and C (TGCTGCTGCTGCTGCGTAACCGTATCTAACCAATAA),

D (ATCGATGGTACCCTTCCAGTATCAGTGGGTTC), respectfully, were used to make a fused product by overlap PCR using primers A and D (Horton et al., 1989). For both strains, the PCR product was cloned in the allelic exchange vector pRE107 (Edwards et al., 1998) in *E. coli* K12 strain B2155, transferred to parental strains by conjugation, and recombinants selected on TB medium containing 100  $\mu$ g/ml ampicillin with no diaminopimelic acid. Following counter-selection with 5% sucrose, deletion mutants were identified by PCR. KIM5 $\Delta$ YopJ was as described (Vladimer et al., 2012). Fully virulent KIM1001, and the attenuated strains KIM5-EcLpxL and KIM1001-EcLpxL, expressing pBR322 containing *E. coli* lpxL and constitutively generating a TLR4-stimulating hexa-acylated lipid A, were as described (Montminy et al., 2006).

### **Cell stimulations**

Mouse bone marrow-derived macrophages (BMDM) were prepared by maturing bone marrow cells for 6-7 days in the presence of M-CSF-containing supernatant from L929 cells. Some cell stimulations were also conducted with peritoneal macrophages and immortalized BMDM, and similar results to stimulations done with BMDM were obtained. RIP1<sup>+/+</sup> and RIP1<sup>-/-</sup> immortalized fetal liver macrophages were provided by M. Kelliher and K. Fitzgerald. Mouse peritoneal

macrophages were harvested from mice 4 days after injection of 3 ml thioglycollate (injected by i.p.). Mouse macrophages were plated at a density of  $5 \times 10^4$  cells per well in 96-well dishes and incubated overnight. Bacterial infections were performed at the MOIs indicated and gentamicin ( $40 \mu\text{g/mL}$ ) was added 1 hr post-infection. Supernatants were collected for cytokine analysis or LDH assays at the indicated time points. In some experiments, cells were pre-treated with  $1 \mu\text{M}$  of the RIP1 inhibitor GSK'963 or its inactive enantiomer GSK'962 (P.A.H., J.B., P.J.G., manuscript in preparation),  $3 \mu\text{M}$  of the RIP3 inhibitor GSK'872,  $20 \mu\text{M}$  Nec-1 (Enzo Life Sciences),  $20 \mu\text{M}$  IETD, YVAD or zVAD (Promega) 1 hour prior to infection.  $10 \mu\text{M}$  Nigericin (Sigma-Aldrich),  $10 \text{ ng/ml}$  of hexa-acylated LPS from *Y. pestis* grown at  $26^\circ\text{C}$  or from *E. coli*, and  $300 \text{ ng/ml}$  Pam3CysSK4 (Pam3Cys, Invitrogen) were used as controls. Cytokine levels and caspase-1 cleavage were measured by ELISA and western blot following the manufacturer's instructions or as previously reported (Vladimer et al., 2012). ELISA antibodies or kits were from R&D systems (mouse TNF, IL-6, IL-18 and IL- $1\beta$ ). Caspase-8 activity (Promega) was measured 2 hrs post infection.

### **Western blot analysis**

Protein from cell supernatants was precipitated by methanol-chloroform extraction. Adherent cells were lysed with 1% NP-40 lysis buffer (10% glycerol, 1% NP-40, 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 2 mM EDTA, containing protease inhibitors: 1 mM sodium orthovanadate, 1 mM PMSF and cOmplete Protease Inhibitor Cocktail (Roche). Immunoblot analysis was mainly as previously described (Vladimer et al., 2012) with antibodies to mouse caspase-8 (clone: 1G12, Enzo Life Sciences), caspase-3 (clone: 5A1E, Cell Signaling), mouse caspase-1 p20 (clone: 5B10, eBioscience), mouse IL- $1\beta$  (catalog #: AF-401-NA, R&D Systems), RIP1 (clone: 38/RIP, BD Transduction Laboratories), I $\kappa$ B $\alpha$  (catalog #: 9242, Cell Signaling), phospho-I $\kappa$ B $\alpha$  (catalog #:

2859, Cell Signaling),  $\beta$ -actin (BD Pharmingen), RIP3 (catalog #: 2283, ProSci), p65 (Cell Signaling) or Histone 3 (Abcam).

### **LDH assay**

Cell death was measured by detecting the lactate dehydrogenase (LDH) release using the CytoTox-96 nonradioactive cytotoxicity kit (Promega) according to the manufacturer's instructions. Background LDH release was determined by assaying supernatants from uninfected cells, and total LDH was determined from uninfected cells which had been lysed by lysis buffer. The percentage of cell death was calculated as:  $(\text{sample LDH} - \text{background LDH}) / (\text{total LDH} - \text{background LDH}) \times 100\%$ .

### **FACS analysis**

Single-cell suspensions of splenocytes from infected animals or mouse macrophages were treated with Fc block (clone 93, rat anti-CD16/CD32, eBiosciences) for 15 minutes on ice, then stained with the indicated antibodies: CD40-APC (clone:1C10, eBiosciences) and CD11b-PE (clone:M1/70, eBiosciences). Where applicable, dead cells were enumerated using the live/dead blue stain (Invitrogen) or propidium iodide (PI staining solution, BD) according to the manufacturer's instructions. Cells from infected mice were fixed with 2% formaldehyde prior to analysis by flow cytometry. Data acquisition was performed with an LSRII instrument (BD) and BD FACSDiva™ software, and analysis was conducted with FlowJo.

### **Caspase-8 activation assay**

To determine the status of caspase-8 enzymatic activity,  $1 \times 10^5$  cells were plated into each well of a 96-well plate and stimulated with *Y. pestis* at an MOI40 for 2 hours. Caspase-8 activation in cell

lysates was detected using Caspase-Glo® 8 Assay (Promega) according to the manufacturer's instructions.

#### **qPCR analysis of Il1b expression in primary mouse macrophages**

RNA from resting or stimulated (with *Y. pestis* or LPS) BMDM was purified with the RNeasy Mini Kit (Qiagen) and cDNA was generated with the iScript cDNA Synthesis Kit (BioRad). PCR was performed as reported (Vladimer et al., 2012) on transcribed cDNA with primers for detection of mouse Il1b

(5'-GCCCATCCTCTGTGACTCAT-3', 5'-AGGCCACAGGTATTTTGTCG-3') using SYBR green (BioRad) according to the manufacturer's instructions.

#### **Staining and microscopy**

Tissue samples for histology were fixed in 10% buffered formalin for a minimum of 72 hours with 3 changes of fixative. Hematoxylin and eosin (H&E) staining and microscopy were performed as previously described (Montminy et al., 2006; Vladimer et al., 2012).

#### **Electron microscopy**

Wild-type, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup>, or RIP3<sup>-/-</sup> immortalized mouse macrophages were infected with *Y. pestis* KIM5 for 2.5 hours and subjected to electron microscopy analysis by the UMass Medical School Core Electron Microscopy Facility. Cells were fixed for 10 minutes by adding 1 ml of 2.5% glutaraldehyde (v/v) in 0.75 M sodium phosphate buffer (pH 7.2) to each of the medium-containing wells of the culture plate. Fixative was removed and added at full strength overnight at 4°C. The next day the fixed samples were washed three times in 0.75 M sodium phosphate buffer (pH 7.2). The cells were then scraped off the bottom of the wells with a soft plastic spatula,

collected in a microcentrifuge tube, pelleted, briefly rinsed in deionized water, post-fixed for 1 hr in 1% osmium tetroxide (w/v) in deionized water, washed, and dehydrated through a graded series of ethanol concentration increases in 20% increments before two changes in 100% ethanol. Samples were then infiltrated first with two changes of propylene oxide and then a mixture of 50% propylene oxide / 50% SPIpon 812/Araldite epoxy resin overnight. Cell pellets were transferred through three changes of fresh SPIpon 812/ Araldite epoxy resin, embedded into molds filled with the same resin, and polymerized for 48 hrs at 70°C. The epoxy blocks were then trimmed and ultrathin sections were cut on a Reichart-Jung ultramicrotome using a diamond knife. The sections were collected and mounted on copper support grids and contrasted with lead citrate and uranyl acetate. The samples were examined on a FEI Tecnai 12 BT transmission electron microscope using 80Kv accelerating voltage. Images were captured using a Gatan TEM CCD camera.

### **Statistical analysis**

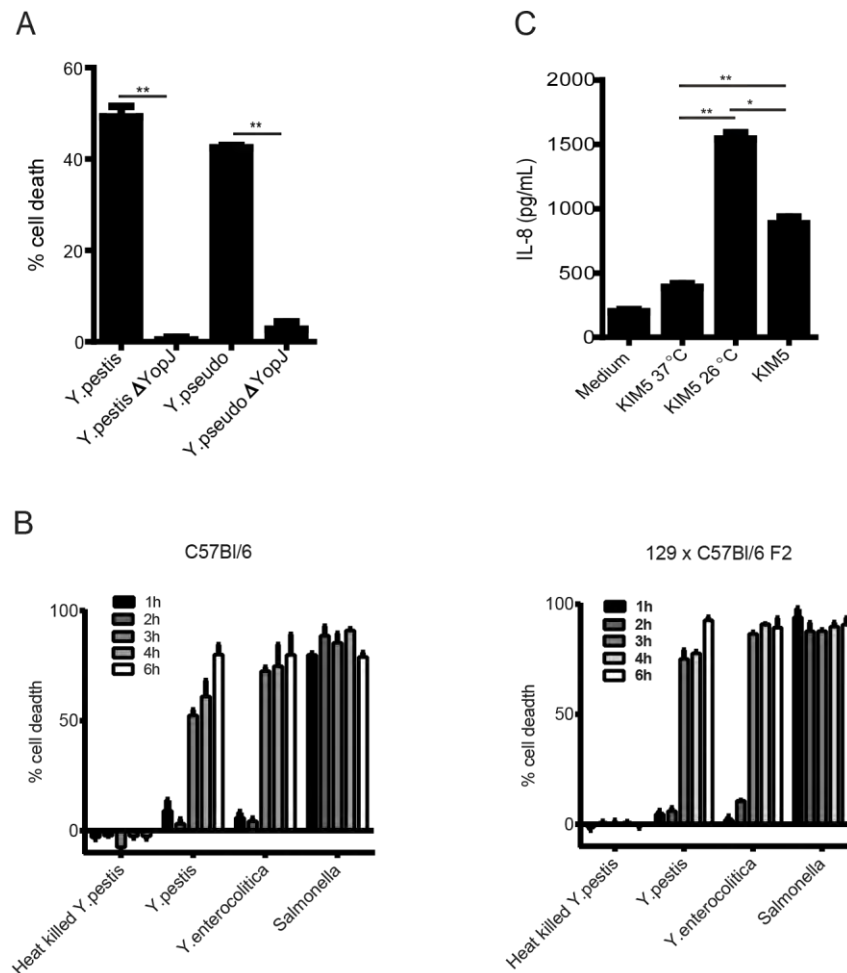
Statistical analysis was performed with GraphPad Prism® 5.0. To evaluate the differences between two groups (cell death and BMDM cytokine release), the two-tailed t-test was used. When more than two groups were compared, two-way ANOVA with Tukey's post-test was utilized. Differences between cytokine levels in infected mice were analyzed by the Mann-Whitney U test. Group size for animal experiments was calculated by power analysis. Survival analysis was performed using the log-rank test.

## Results and Discussions

### ***Yersinia* induces cell death via RIP1, caspase-8 and RIP3**

Viable *Y. pestis* KIM5 can induce rapid cell death (Fig. 2.1A). Cell death in bone marrow-derived macrophages (BMDMs) is induced in a YopJ-dependent manner by *Y. pestis* or *Y. pseudotuberculosis* temperature-shifted from 26°C to 37°C (Fig. 2.1A,B), a condition that mimics the temperature change from flea temperature (26°C) to mammalian body temperature (37°C) which is associated with infection via a fleabite. In addition to arming the type three secretion system (T3SS), the temperature shift ensures the initial presence of some TLR4-stimulatory LPS which is hexa-acylated lipid A/LPS and is synthesized only at 26°C and not 37°C (Montminy et al., 2006). The TLR4-stimulatory activity of LPS synthesized by *Y. pestis* was measured through the production of cytokine IL-8 by HEK293 cells that stably express human TLR4/MD2 (Fig. 2.1C).

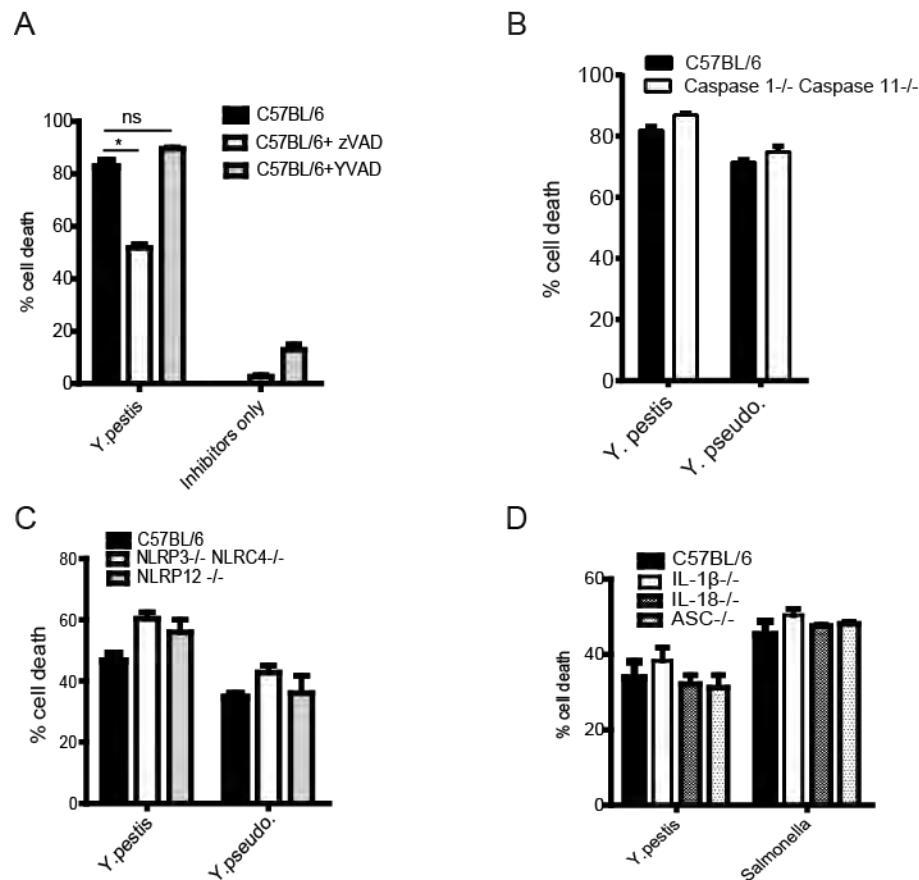
*Y. pestis* can induce inflammasome activation and caspase-1 cleavage (Lilo et al., 2008; Vladimer et al., 2012). As active caspase-1 induced by inflammasome activation also leads to pyroptotic cell death, we investigated whether *Y. pestis*-induced cell death is due to pyroptosis. First, the BMDMs were pretreated with the caspase-1 inhibitor YVAD and the results indicated that YVAD did not abrogate *Y. pestis*-induced cell death, suggesting that caspase-1 activity was not required in *Yersinia* cytotoxicity (Fig. 2.2A). Using caspase-1<sup>-/-</sup>caspase-11<sup>-/-</sup> BMDMs, it was further confirmed that macrophage death induced by *Y. pestis* was independent of caspase-1 and caspase-11, thus the mode of cell death is not through pyroptosis (Fig. 2.2B). In addition to caspase-1, the inflammasome-related NLRs, NLRP3, NLRC4 and NLRP12 are also not involved



**Figure 2.1 Live *Yersinia* induces rapid cell death that is dependent on YopJ and *Y. pestis* shows TLR4-stimulating activity when grown at different temperatures.**

(A) BMDMs were stimulated with the indicated *Yersinia* strains (MOI 40) for 4 h, and cell death was determined by LDH assay. BMDMs were killed by *Y. pestis* and *Y. pseudotuberculosis*, but not by bacteria lacking the T3SS effector YopJ. (B) C57Bl/6 or 129xC57Bl/6 F2 immortalized macrophages were infected with live *Y. pestis*, *Y. enterocolitica* (MOI 40), *Salmonella* (MOI 1.5), or heat killed (56°C, 2 h) *Y. pestis* (equivalent to MOI of 40) for the indicated time points. Cell death is comparable between C57Bl/6 cells and 129 x C57Bl/6 F2 macrophages. (C) HEK293 cells that stably express human TLR4/MD2 were stimulated with heat-killed *Y. pestis* KIM5 (MOI 10) for 18 h. KIM5 37°C bacteria were grown continuously at 37°C, and KIM5 26°C were grown continuously at 26°C before being heat-killed. IL-8 in supernatant was detected by ELISA. Figures are representative for 3-8 experiments performed. Bars indicate mean plus SD. \*\*p<0.01, \*p<0.05 (two-tailed t-test)



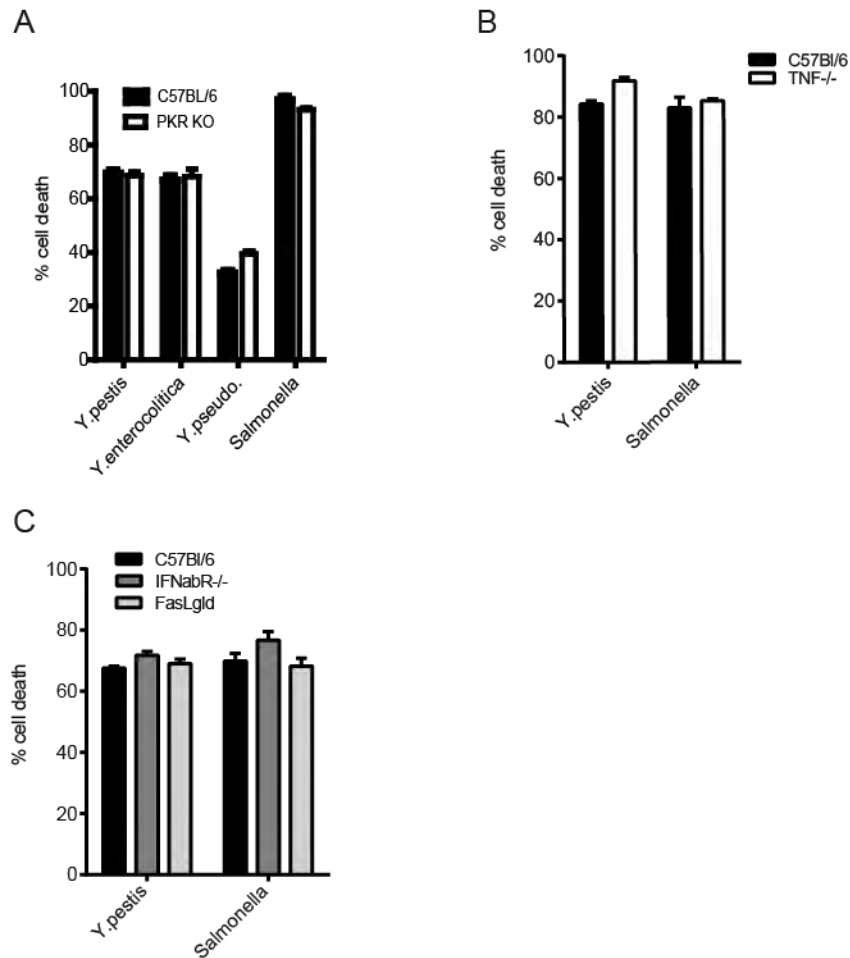


**Figure 2.2** *Yersinia*-induced macrophage death does not require caspase-1, caspase-11, NLRP3, NLRC4, NLRP12, IL-1 $\beta$ , IL-18 or ASC.

A-D) BMDMs were infected with *Y. pestis*, *Y. pseudotuberculosis* (MOI 40) or *Salmonella* (MOI 1.5) for 4 hrs as indicated. In (A) cells were pretreated for one hr prior to infection with 20  $\mu$ M of the pan-caspase inhibitor zVAD-fmk or the caspase-1 inhibitor YVAD-fmk. Cell death was determined by measuring LDH release into the supernatant. Figures are representative for 3-5 experiments performed. Bars indicate mean plus SD. \* $p < 0.05$  (two-tailed t-test)

in *Y. pestis*-induced cell death (Fig. 2.2C). Similarly, deficiency of the inflammasome adaptor ASC, cytokines IL-1 $\beta$  or IL-18 failed to influence the cytotoxicity of *Y. pestis*, again confirming that *Y. pestis*-induced cell death is not inflammasome-related pyroptosis (Fig. 2.2D). Although the RNA-dependent protein kinase (PKR) has been suggested to contribute to *Yersinia pseudotuberculosis*-induced cell death (Hsu et al., 2004), our results demonstrate that *Y. pestis*-induced cell death between wild type and PKR<sup>-/-</sup> BMDMs is comparable (Fig. 2.3A). The difference might be due to the use of different cell types, different bacterial strains and infection conditions. Additionally, *Y. pestis*-induced cell death was unaffected by a deficiency in Fas ligand (FasL), TNF- $\alpha$ , or IFN $\alpha$ R, indicating that those death receptor-mediated cell death pathways are not involved (Fig. 2.3BC), which is consistent with previous studies with *Y. enterocolitica* YopP (Gröbner et al., 2007a). Therefore, although *Y. pestis* can induce TNF $\alpha$  production, cell death induced is not due to the cytotoxicity outcome of autocrine TNF $\alpha$  production.

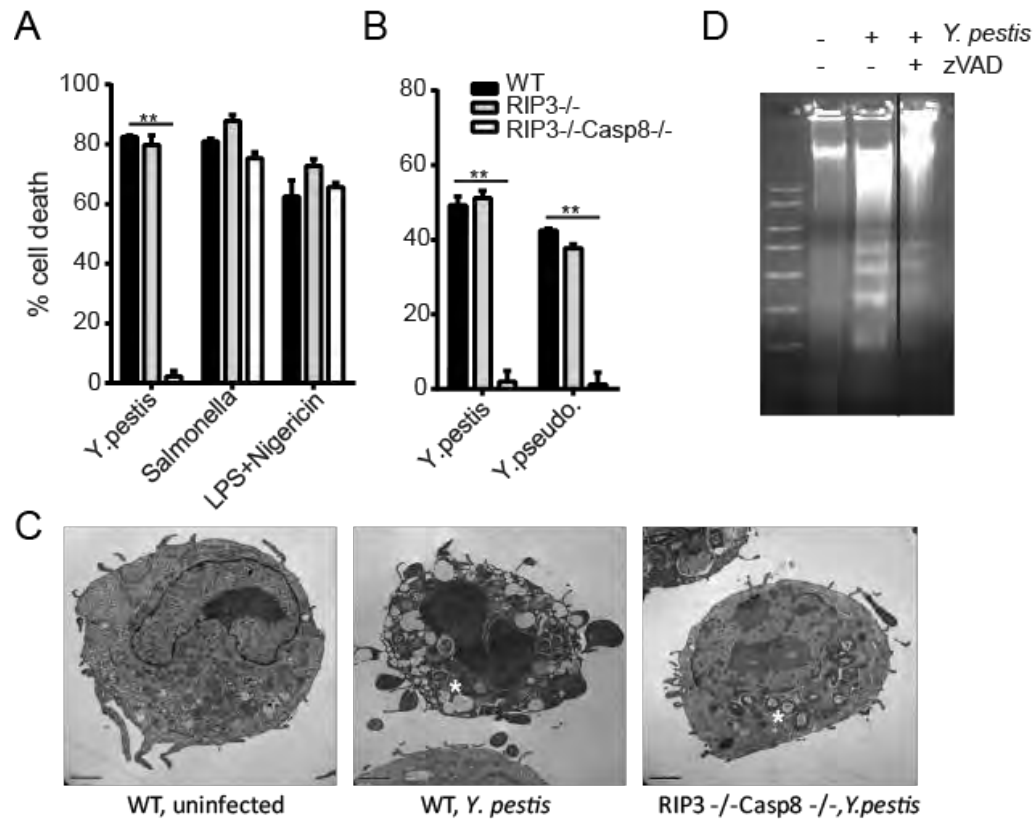
Pretreatment of BMDMs with the pan-caspase inhibitor zVAD-fmk partially inhibits *Y. pestis*-induced cell death (Fig. 2.2A), suggesting the involvement of caspases other than caspase-1. It has been shown that *Yersinia* YopJ/YopP induces the activation and processing of several caspases, including caspase-8, -3 and -7 (Denecker et al., 2001; Gröbner et al., 2007a; Monack et al., 1998). Caspase-8 is a key initiator caspase in extrinsic apoptosis induced by death receptors and some viruses (Oberst and Green, 2011), and its activation leads to the processing of the executioner caspases, caspase-3 and -7. Caspase-8 deficiency results in embryonic lethality, but mice deficient in both caspase-8 and RIP3 (RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice, DKO) are rescued (Kaiser et al., 2011; Oberst et al., 2011). These studies indicate a vital role for caspase-8 in suppressing necroptosis by targeting components of the RIP3-mediated necrosis pathway (Kaiser et al., 2011; Oberst et al., 2011). The availability of RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice makes it possible to study the outcome of genetic deficiency of caspase-8 in different diseases or microbial infections. To investigate whether caspase-8 is required for *Y. pestis*-induced cell death, we used macrophages from RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice, which were remarkably resistant to cell death induced by *Y. pestis*



**Figure 2.3** TNF $\alpha$ , IFN $\alpha$ B $\beta$ R1, FasL or PKR are dispensable for *Yersinia*-induced macrophage death.

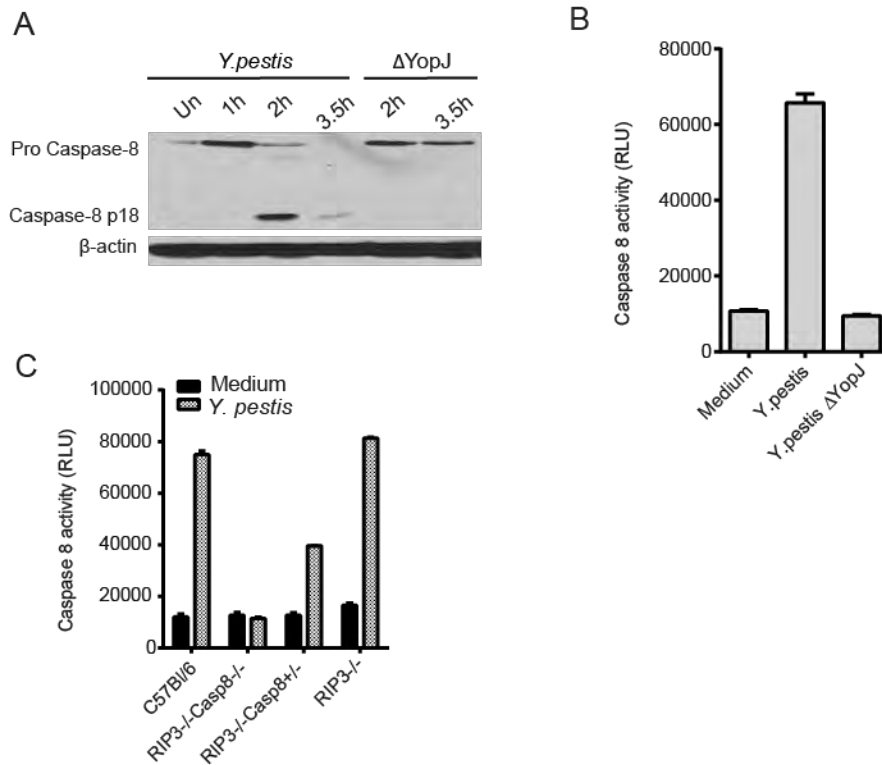
A-C) BMDMs were infected with *Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis* (MOI 40) or *Salmonella* (MOI 1.5) for 4 hrs as indicated. Cell death was determined by measuring LDH release into the supernatant. Figures are representative for 3 experiments performed. Bars indicate mean plus SD.

and *Y. pseudotuberculosis*, but not by *Salmonella* which induces pyroptotic cell death (Miao et al., 2010), or by the NLRP3 inflammasome-specific trigger nigericin (Fig. 2.4AB). Although previous studies suggested that *Yersinia*-infected cells exhibit both apoptotic and necrotic properties, in contrast to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, RIP3-deficient cells are not protected from YopJ-induced cell death (Fig. 2.4AB). This finding suggests that *Y. pestis*-induced cell death is likely not necroptosis or that necrosis mediated by RIP3 is suppressed when caspase-8 is present. Electron microscopy revealed that macrophages infected with *Y. pestis* displayed features consistent with apoptotic death, such as membrane blebbing, nuclear condensation and fragmentation. These effects were absent in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages which are visibly infected with intracellular *Y. pestis* (white arrows, Fig. 2.4C). Moreover, infection of macrophages led to DNA fragmentation patterns which are typically associated with apoptosis, and this fragmentation was blocked by zVAD pan-caspase inhibition (Fig. 2.4D). Taken together, these data strongly suggest that *Yersinia* induces rapid macrophage death by apoptosis via caspase-8. Consistent with previous reports, *Y. pestis* induced the cleavage of caspase-8 at 2 hours post of infection, resulting in the production of caspase-8 p18 subunit, and this cleavage is YopJ-dependent (Fig. 2.5A). Similar results were observed by quantitatively measuring caspase-8 enzymatic activity. Wild type *Y. pestis* induced robust caspase-8 activation while the YopJ mutant strain did not (Fig. 2.5B). The strong caspase-8 activity induced by *Y. pestis* was approximately 50% lower in RIP3<sup>-/-</sup>caspase-8<sup>+/-</sup> macrophages compared with wild type cells, and caspase-8 activation was completely abrogated in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.5C). This data is in agreement with the genetic deficiency of caspase-8 in these cells, indicating that this caspase-8 enzyme assay is a reliable and consistent approach to quantitatively detect the activation and enzymatic activity of caspase-8 (Fig. 2.5C). In most, but not all situations, RIP3-mediated necroptotic death also requires RIP1 (Cho et al., 2009; Moquin and Chan, 2010; Zhang et al., 2009), a serine/threonine kinase that also contributes to NF- $\kappa$ B signaling



**Figure 2.4 Caspase-8-RIP3 deficient macrophages are protected against *Y. pestis*-induced cytotoxicity.**

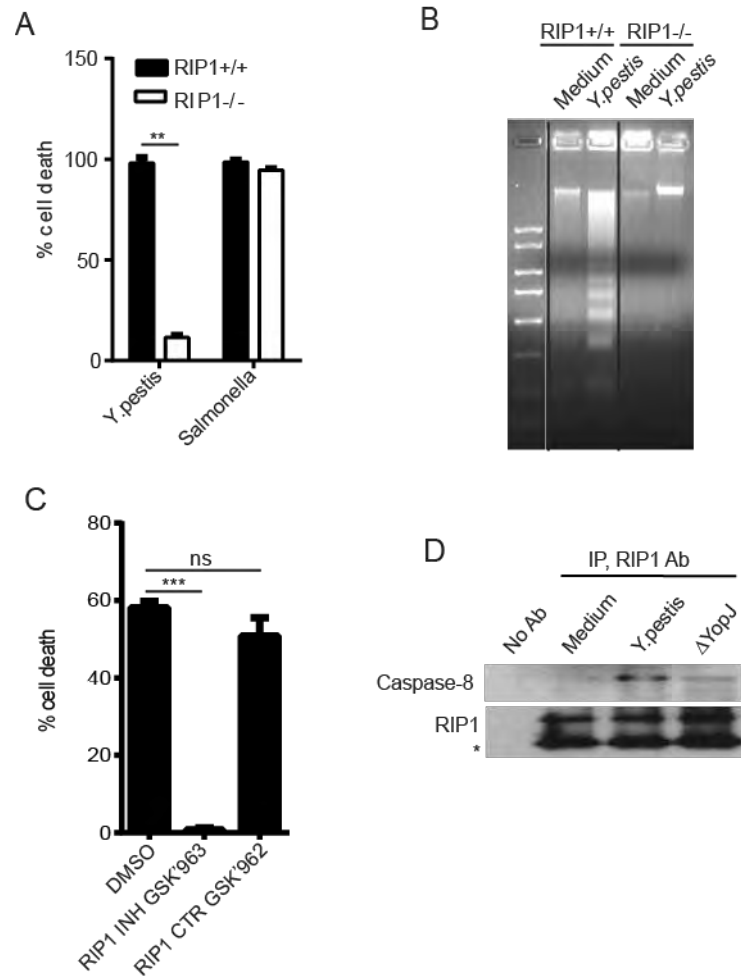
(A-B) Caspase-8<sup>-/-</sup> RIP3<sup>-/-</sup> (DKO), but not RIP3 KO BMDMs are protected from *Yersinia*-induced cytotoxicity measured by LDH release assay or C) electron microscopy. Bar=2μm. White asterisks: bacteria. D) WT immortalized macrophages were treated with zVAD (20 μM) for 1 hr before *Y. pestis* infection (MOI 40, 3 h). A Calbiochem Suicide-track DNA ladder isolation kit was used to detect DNA fragmentation according to the manufacturer's instructions. BMDMs were infected with 10-40 multiples of infection (MOI) of *Yersiniae* or 1.5 MOI of *S. typhimurium* for 4 hrs (A,B) or 2 hrs (C), and gentamycin was added after 1 hr. Figures are representative for 3-8 experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)



**Figure 2.5** *Y. pestis* induces caspase-8 cleavage and activation, which is dependent on the presence of YopJ.

(A-B) C57Bl/6 BMDMs were infected with *Y. pestis* or *Y. pestis*  $\Delta$ YopJ (MOI 40) for indicated time points (A) or 2 hrs (B). The cleavage of caspase-8 was detected by western blot (A) and the caspase-8 activation by Caspase-Glo 8 assay (B). (C) BMDMs were infected with *Y. pestis* (MOI 40) for 2 hrs and the caspase-8 activity was measured by Caspase-Glo 8 assay. Figures are representative for 3-5 experiments performed. Bars indicate mean plus SD.

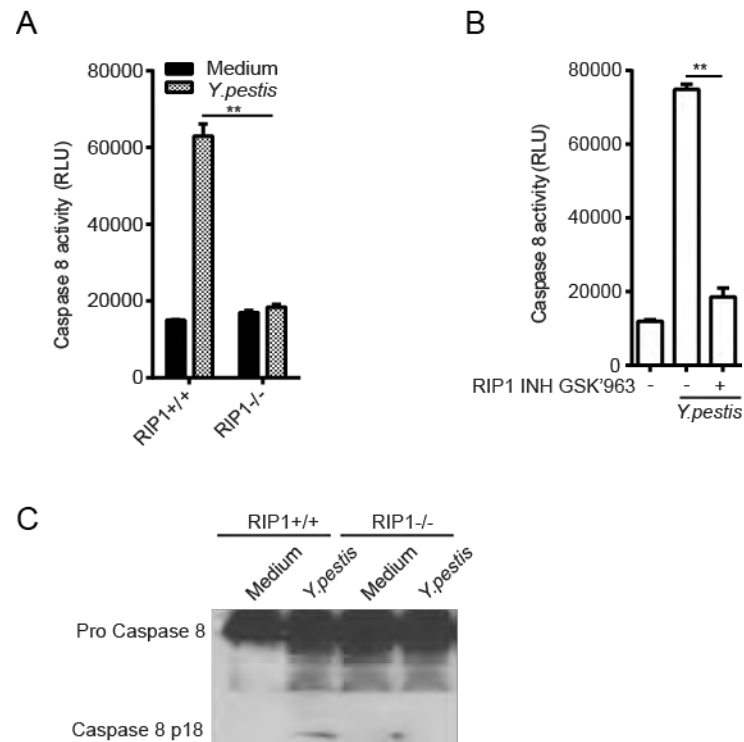
(Cusson-Hermance et al., 2005) and apoptosis (Christofferson et al., 2014). RIP1 is a critical regulator and decision checkpoint for TNF-induced NF- $\kappa$ B activation, apoptosis or necrosis through its kinase and scaffolding activities (Festjens et al., 2007). In addition to death receptors, RIP1 also regulates cell survival and death fate decisions downstream of TRIF through RHIM domain interactions between TRIF and RIP1 when responding to TLR3 or TLR4 ligands (Feoktistova et al., 2011). Grobner et al. suggested the potential involvement of RIP1 in the cell death induced by *Y. enterocolitica* YopP (Gröbner et al., 2007a). We therefore investigated the potential requirement of RIP1 in *Y. pestis*-induced cell death. RIP1<sup>-/-</sup> mice die shortly after birth (Kelliher et al., 1998), but fetal liver macrophages from RIP1<sup>-/-</sup> mice, in contrast to RIP3<sup>-/-</sup> macrophages, displayed a complete rescue from cell death induced by *Y. pestis* (Fig. 2.6A). Furthermore, DNA fragmentation induced by *Y. pestis* infection, was absent in RIP1<sup>-/-</sup> fetal liver macrophages (Fig. 2.6B). A novel, potent and specific inhibitor of RIP1 kinase activity, has recently been identified (P.A.H., J.B., P.J.G., manuscript in preparation). The RIP1 inhibitor GSK'963, but not the inactive enantiomer GSK'962, blocks *Y. pestis*-induced cell death (Fig. 2.6C). Taken together, the results by both genetic evidence and pharmaceutical inhibitor suggest that RIP1 is also required for *Y. pestis*-induced apoptotic cell death. Since RIP1 has been suggested to interact with caspase-8 and FADD to mediate the apoptosis induced by TNF, or cIAP inhibitors (Bertrand and Vandenabeele, 2011; Tenev et al., 2011; Vince et al., 2007), we therefore propose that RIP1 and caspase-8 are involved in the same pathway to mediate *Y. pestis*-induced apoptosis. To evaluate this hypothesis, we used co-IP to detect the *Y. pestis*-induced interaction between RIP1 and caspase-8 (Fig. 2.6D). *Y. pestis*  $\Delta$ YopJ failed to induce the interaction of RIP1 and caspase-8, consistent with the inability of the YopJ mutant strain to induce cell death (Fig. 2.1A). In addition, *Y. pestis*-induced robust caspase-8 enzymatic activation was totally suppressed in RIP1<sup>-/-</sup> cells or significantly decreased by RIP1 kinase inhibitor GSK'963 pretreatment (Fig. 2.7AB). Accordingly, the cleavage of caspase-8 was also inhibited in RIP1<sup>-/-</sup> macrophages (Fig. 2.7C), suggesting that RIP1 might function upstream of caspase-8 and



**Figure 2.6 RIP1 inhibition or deficiency protects macrophages from *Y. pestis*-induced cell death.**

(A-B) RIP1-deficient fetal liver macrophages are resistant to *Y. pestis*-induced apoptotic death (MOI 40, 4h), determined by LDH release (A) or DNA fragmentation assay (B). C) WT BMDMs were treated with the RIP1 inhibitor GSK'963 or inactive control compound GSK'962 or vehicle control DMSO for 1 h before *Y. pestis* infection (MOI 40, 4 h), then cell death was detected by LDH assay. D) RIP1 forms a complex with caspase-8 upon infection (1 h), measured by co-IP. Asterisk: IgG heavy chain. Figures are representative for 3-4 experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)



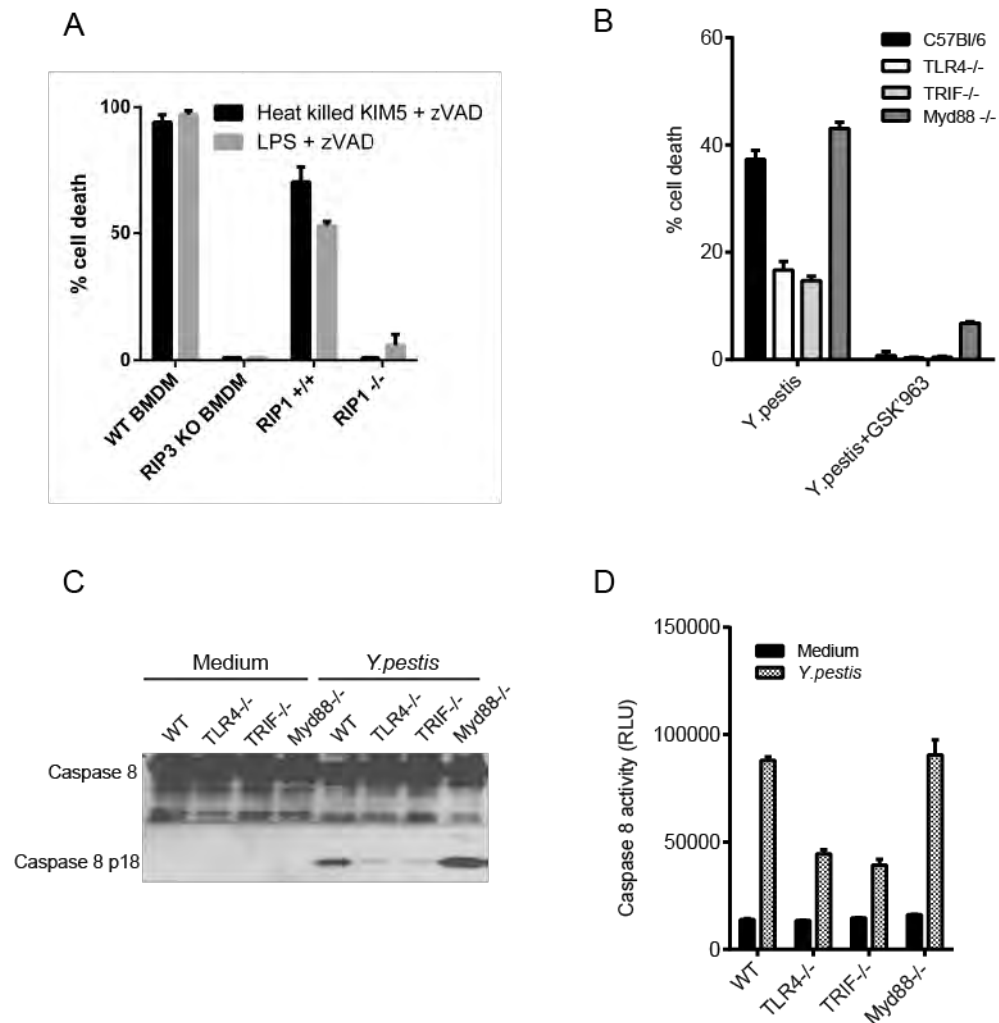


**Figure 2.7** *Y. pestis*-induced caspase-8 activation or cleavage is inhibited in RIP1 deficient fetal liver macrophages or by RIP1 inhibitor.

A, C) Fetal liver macrophages were infected with *Y. pestis* (MOI 40, 2 h). B) C57Bl/6 BMDMs were pretreated with RIP1 inhibitor GSK'963 for 1 h before *Y. pestis* infection (MOI 40, 2 h). Then the caspase-8 activity was measured by Caspase-Glo 8 assay (A, B) or the cleavage of caspase-8 was detected by Western blot (C). Shown is representative of three performed experiments. Bars indicate mean plus SD. \*\* $p < 0.01$  (two-tailed t-test)

regulate caspase-8 activation to trigger apoptosis.

Previous reports indicate that TLR4<sup>-/-</sup> BMDMs are partially protected from *Yersinia*-induced cytotoxicity (Gröbner et al., 2007b; Zhang and Bliska, 2003). Downstream of TLR4, TRIF<sup>-/-</sup> BMDMs show a similar protective effect as TLR4 deficient cells. In contrast, MyD88<sup>-/-</sup> BMDMs exhibit cell death similar to wild type cells (Fig. 2.8B). TLR4 signaling can also induce necroptosis when cells are treated with zVAD-fmk to inhibit caspase-8 via a TRIF- and RIP3-dependent pathway (He et al., 2011; Kaiser et al., 2013). Our data supports the conclusion that LPS plus zVAD induced a RIP3-dependent necrosis in macrophages (Fig. 2.8A). In addition, heat-killed *Y. pestis* showed a similar effect as LPS to induce RIP3-dependent necrosis when combined with zVAD to stimulate cells (Fig. 2.8A). Moreover, RIP1<sup>-/-</sup> fetal liver macrophages were also protected from necroptotic cell death induced by LPS plus zVAD or heat killed *Yersinia* plus zVAD (Fig. 2.8A). To further investigate the role of the TLR4 signaling pathway, we infected TLR4<sup>-/-</sup>, TRIF<sup>-/-</sup> or MyD88<sup>-/-</sup> BMDMs with *Y. pestis* (MOI 40) for 4 hours and detected LDH released from dying cells. In agreement with studies by other groups (Gröbner et al., 2007b; Zhang and Bliska, 2003), *Y. pestis*-induced BMDM death is partially dependent on TLR4 and TRIF, but not MyD88 (Fig. 2.8B). RIP1 kinase inhibition by GSK'963 further suppressed the remaining cell death of TLR4<sup>-/-</sup> and TRIF<sup>-/-</sup> BMDMs (Fig. 2.8B), suggesting that RIP1 contributes to both TLR4-TRIF-mediated and TLR4-TRIF-independent cell death pathways. Accordingly, cleavage of caspase-8, as well as caspase-8 activation, was impaired in TLR4- and TRIF-deficient BMDMs but not in MyD88-deficient cells (Fig. 2.8CD). Reduced cell death in TRIF<sup>-/-</sup> BMDMs was also seen for bacteria grown at 37°C and *Y. pestis*-EcLpxL, which constitutively generates a hexa-acylated LPS even when grown at 37°C (Fig. 2.9B). Based on these results, TLR4-TRIF signaling appears to not be absolutely required, but enhances caspase-8

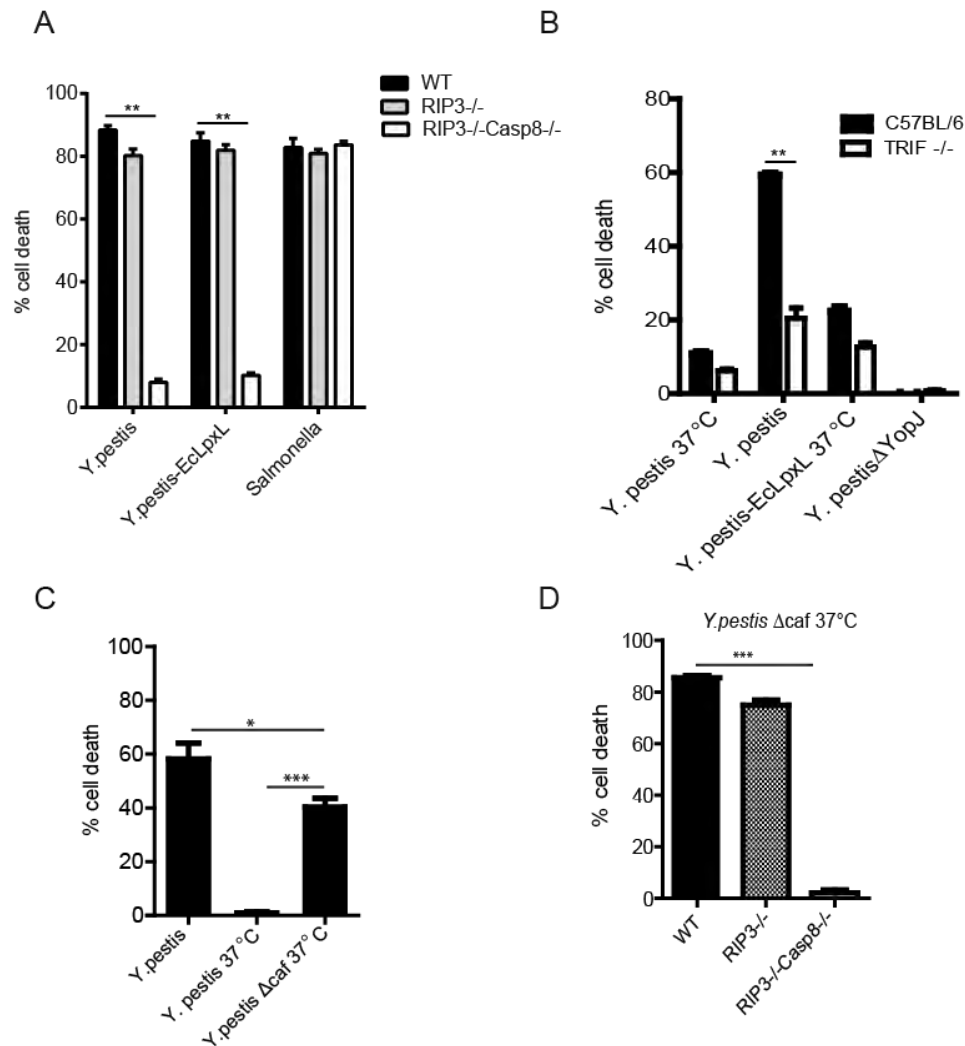


**Figure 2.8 *Y. pestis*-induced macrophage death, caspase-8 cleavage and activation partially require TLR4 and TRIF but not MyD88.**

A) Necroptosis induced by zVAD (20  $\mu$ M) plus LPS (50 ng/mL) or zVAD plus heat-killed *Y. pestis* KIM5 (equal to MOI 10) (18 h stimulation) is greatly decreased in RIP3<sup>-/-</sup> BMDMs or RIP1<sup>-/-</sup> fetal liver macrophages. B-D) BMDMs from WT, TLR4<sup>-/-</sup>, TRIF<sup>-/-</sup> or MyD88<sup>-/-</sup> mice were infected with *Y. pestis* (MOI 40) for 2 (C,D) or 4 hrs (B). In (B), cells were pretreated for 1 hour before infection with the RIP1 inhibitor GSK'963. Supernatants were assayed for LDH release to determine cell death. Caspase-8 cleavage by Western blot (C) and caspase-8 activity by Caspase-Glo 8 assay (D). B) *Y. pestis*-induced BMDM death is partially dependent on TLR4 and TRIF but not MyD88 that is further reduced when RIP1 is inhibited. C) Cleavage of caspase-8 is impaired in TLR4<sup>-/-</sup> and TRIF<sup>-/-</sup> BMDMs but not in MyD88<sup>-/-</sup> BMDMs. D) Caspase-8 activity induced by *Y. pestis* is impaired in TLR4<sup>-/-</sup> and TRIF<sup>-/-</sup> BMDMs but not in MyD88<sup>-/-</sup> BMDMs. Shown is representative of three performed experiments. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)

-mediated apoptosis by *Y. pestis* YopJ, similar to that proposed for *Y. enterocolitica* YopP (Gröbner et al., 2007b; Haase et al., 2003; Zhang and Bliska, 2003).

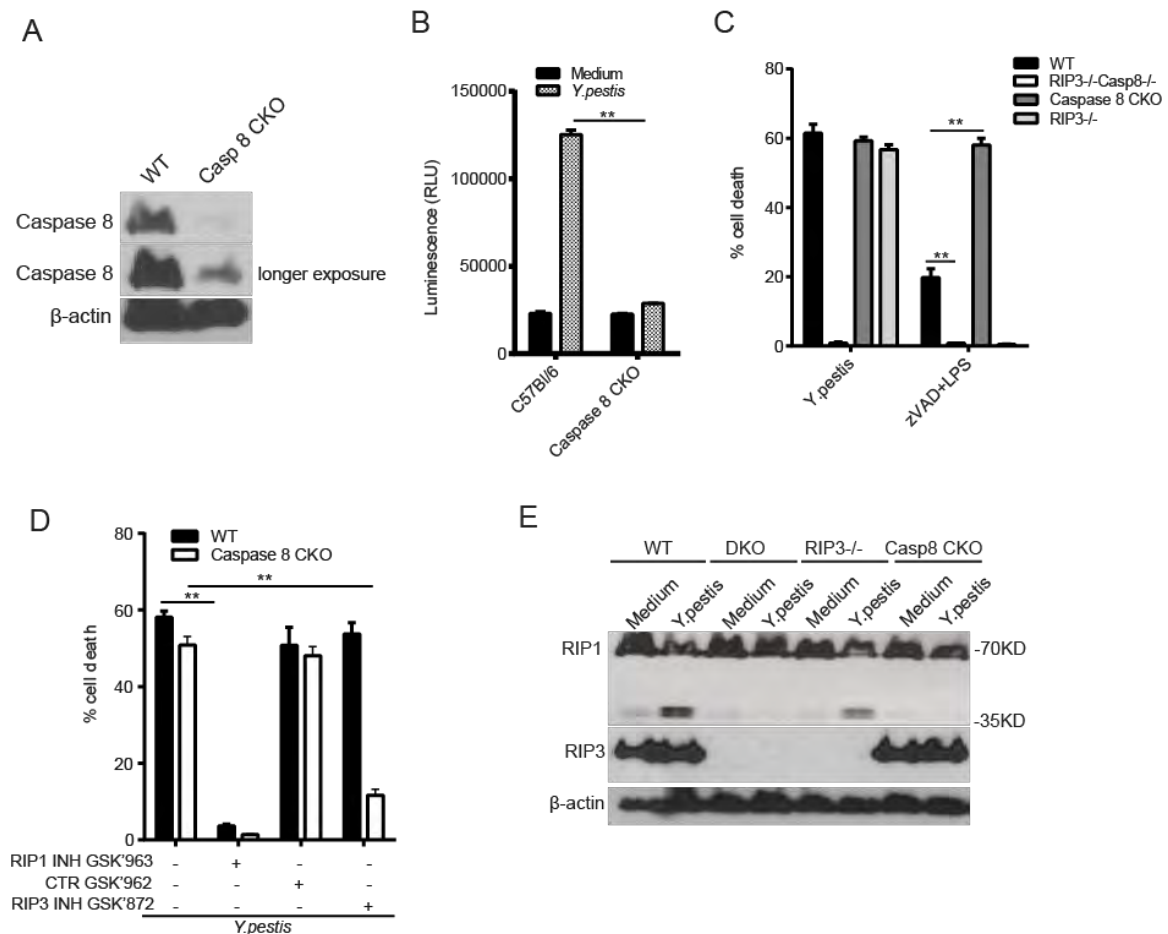
We also evaluated other mutant strains of *Y. pestis* previously made by our group to determine whether they utilize the same cell death pathway as wild type *Y. pestis*. As shown in Fig. 2.9A, the *Y. pestis*-EcLpxL strain, which stably expresses *E. coli* LpxL and thus synthesizes stimulatory LPS at 37°C (Montminy et al., 2006), triggers a RIP3/caspase-8-dependent cell death similar to wild type *Yersinia*. The assembly of the T3SS injectisomes occurs only at 37°C and the activation of T3SS to translocate the Yop effectors into host cells requires bacteria and host cell contact. However, when *Y. pestis* is grown continually at 37°C, it produces a protein “capsule” F1 antigen to inhibit phagocytosis thus preventing its contact with the host cell (Du, 2002; Liu et al., 2006). Accordingly, *Y. pestis* which continuously grown at 37°C failed to induce robust cell death as the temperature-shifted bacteria even with the presence of potent LPS by expressing *E.coli* LpxL (Fig. 2.9B). However, all the bacteria with different growth conditions triggered a decreased cell death in TRIF<sup>-/-</sup> macrophages (Fig. 2.9B), suggesting they induce cytotoxicity via the same mechanisms. Although cell death induced by *Y. pestis* grown at 37°C was inhibited by the presence of CaF1 capsule protein, the mutation of *caf* gene to destroy the expression of this capsule restored bacterial capability to induce cytotoxicity (Fig. 2.9C). This result suggests that close contact between bacteria and host cells, which can be prevented by the capsule, is required for cytotoxicity. Similar as the *E.coli* LpxL expressing strain, this *Y. pestis*  $\Delta$ caf mutant grown at 37°C also induced a RIP3/caspase-8-dependent cell death (Fig. 2.9D).



**Figure 2.9** *Y. pestis* expressing E.coli LpxL strain (*Y. pestis*-EcLpxL) which produces potent LPS also induces RIP3/Caspase-8 dependent cell death as wild type *Y. pestis*. A capsule-deficient strain of *Y. pestis* triggers RIP3/Caspase-8 dependent death when bacteria are grown at 37°C.

A-B) BMDMs were infected with *Y. pestis* KIM5, or KIM5-EcLpxL grown at 37°C (B) or grown as our normal shift rationale (A) for 4 hrs. Then LDH release was detected. C-D) Immortalized mouse macrophages were infected with wild type *Y. pestis* or *Y. pestis* Δcaf1 (F1 antigen, protein capsule) grown at 37°C (MOI 40). C) Caf1 deficiency enables *Y. pestis* grown at 37°C to induce cytotoxicity, in contrast to wild type bacteria. D) RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> immortalized macrophages are resistant to *Y. pestis* Δcaf1-induced cytotoxicity. Cell death was measured by LDH release into the supernatant. Figures are representative for three experiments performed. Bars indicate mean plus SD. \*p<0.05 \*\*p<0.01 (two-tailed t-test)

*Y. pestis*-induced cell death is completely inhibited in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, but is not affected in RIP3-deficient cells, suggesting that the cell death is mediated via caspase-8. Therefore, caspase-8 deficient cells should demonstrate a similar phenotype to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages. To address this, caspase-8 conditional knockout (CKO) macrophages resulting from the targeted deletion of caspase-8 in myeloid cells (caspase-8 fl/fl LysM cre<sup>+/+</sup> generated by D. Shayakhmetov) were used. The deficiency of caspase-8 in these conditional knockout cells was confirmed by detecting the expression level and enzyme activity of caspase-8 upon *Y. pestis* infection (Fig. 2.10AB), and showed very little caspase-8 remaining in these CKO BMDMs (Fig. 2.10A). Interestingly, in comparison with RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, caspase-8 deficiency alone had little effect on *Y. pestis*-induced macrophage death (Fig. 2.10C). Although the generation of other mice with defects in caspase-8 in macrophages has been reported (Kang et al., 2004), our caspase-8 CKO BMDM appeared healthy in culture and did not display increased cell death in the absence of infection (data not shown). GSK'872 is a recently defined potent RIP3 inhibitor to specifically inhibit RIP3 kinase activity (Kaiser et al., 2013). Blockade of RIP3 kinase activity with GSK'872 strongly reduced cell death in caspase-8 CKO macrophages, but had no impact on wild type BMDMs, suggesting that deletion of caspase-8 may promote necroptosis by RIP3 (Fig. 2.10D). As illustrated in our previous results, caspase-8 inhibition by the pan-caspase inhibitor zVAD sensitizes macrophages to RIP1- and RIP3-dependent necroptosis in response to LPS or heat killed bacteria (Fig. 2.8A). Taken together, we propose that RIP1-caspase-8-mediated apoptosis is the dominant type of cell death induced by *Yersinia* YopJ; however, when caspase-8 is deleted or inhibited, RIP3-mediated necroptosis will be unblocked. The inhibitory role of caspase-8 to block necroptosis has been well characterized (Kaiser et al., 2011; Oberst et al., 2011). For the underlying mechanism, it was proposed that caspase-8 inhibits RIP3-mediated necroptosis through cleaving RIP1 and RIP3 to separate their kinase domains from the RHIM



**Figure 2.10 Caspase-8 conditional KO (CKO) macrophages are not protected from *Y. pestis*-induced cell death.**

A) Western blot of BMDMs from the caspase-8 conditional knock-out mouse show a significant reduction in caspase-8 expression. B) BMDMs from the conditional caspase-8 knockout mouse infected with *Y. pestis* (MOI 40) for 2 hours show reduced caspase-8 activity compared to WT cells, using the Caspase-Glo 8 assay. C) BMDMs were treated with *Y. pestis* (MOI 8, 9 hrs) or zVAD (20  $\mu$ M) plus LPS (100 ng/mL) for 10 hrs. zVAD plus LPS can induce necroptosis, which is inhibited when RIP3 is deficient. But in caspase-8 conditional knockout macrophages, the necroptosis induced by zVAD and LPS is significantly increased than wild type cells. *Y. pestis*-induced cell death is blocked in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMDMs but not caspase-8 CKO macrophages. D) Caspase-8 CKO macrophages are protected from *Y. pestis* (MOI 40, 4 hrs) induced death in the presence of RIP1 (GSK'963) or RIP3 (GSK'872) kinase inhibitors, but not by inactive control compound GSK'962. E) RIP1 is cleaved following *Y. pestis* infection (MOI 40, 2 hrs) in a caspase-8 dependent fashion. Figures are representative for three experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)

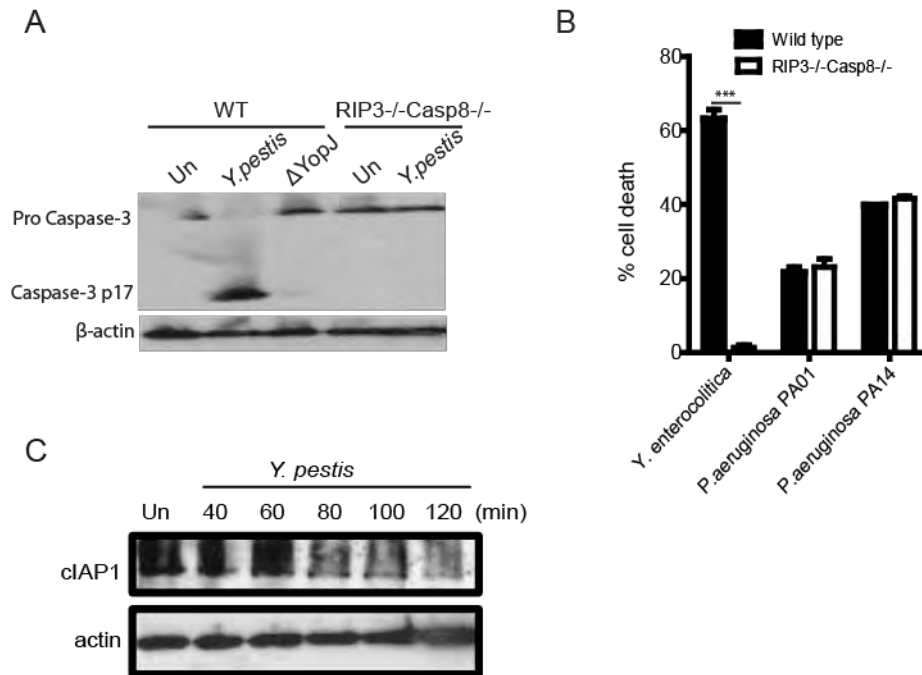
domains (Chan et al., 2003; Feng et al., 2007). Consistent with this proposal, *Y. pestis* induced obviously RIP1 cleavage, which was reduced when caspase-8 was absent (Fig. 2.10E).

As shown in Fig. 2.11A, cleavage and activation of the downstream apoptotic executioner caspase-3 was dependent upon YopJ and caspase-8–RIP3. The caspase-8–RIP3 pathway also influenced cell death induced by *Y. enterocolitica* but not by *Salmonella* or *Pseudomonas*, which also harbor a T3SS (Fig. 2.11B). Thus, all human pathogenic *Yersinia*, but not all bacteria containing a T3SS, trigger cell death via the same pathway. Our results provide an explanation for how *Yersinia* induces macrophage cell death via caspase-8 and RIP kinases. In this model, caspase-8-dependent apoptosis represents the default pathway, while the absence of caspase-8 may lead to RIP3-dependent necroptosis. RIP1 has a key upstream role for both modes of cell death, perhaps influenced by its ability to direct apoptosis under conditions of cIAP1 depletion (Feoktistova et al., 2011; Tenev et al., 2011), also seen with *Y. pestis* infection (Fig. 2.11C).

## Effects on NF- $\kappa$ B activity

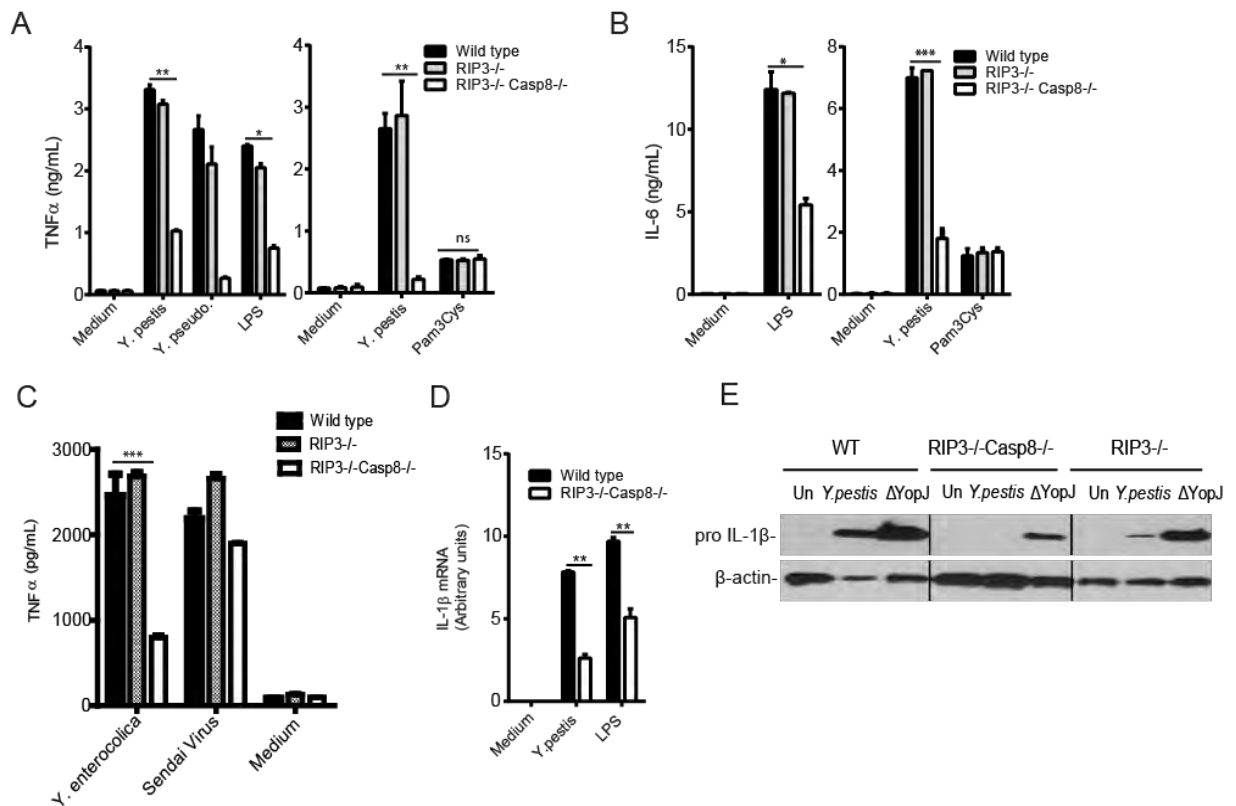
As indicated in Fig 2.12AB, we observed a significant reduction in TNF $\alpha$  and IL-6 release in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup>, but not in RIP3<sup>-/-</sup> macrophages upon *Yersinia* infection or LPS stimulation. However, cytokine production induced by the TLR2 ligand Pam3Cys (Fig. 2.12A,B) or Sendai virus (Fig. 2.12C) was not significantly altered in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages. In addition, IL-1 $\beta$  transcription as well as pro-IL-1 $\beta$  expression, were also attenuated in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages but not in RIP3<sup>-/-</sup> cells (Fig. 2.12DE). The synthesis of TNF $\alpha$ , IL-6 and pro-IL-1 $\beta$  are all regulated by the NF- $\kappa$ B pathway. Caspase-8 has been suggested to have non-apoptotic roles, one of which is to regulate NF- $\kappa$ B activity (Kang et al., 2008; Man et al., 2013; Su et al., 2005). Caspase-8 is required for NF- $\kappa$ B activation by antigen receptor and LPS stimulation, but





**Figure 2.11** *Y. pestis* induced caspase-3 cleavage and cIAP1 depletion and the cell death triggered by *Pseudomonas aeruginosa* is not mediated by RIP3 and caspase-8.

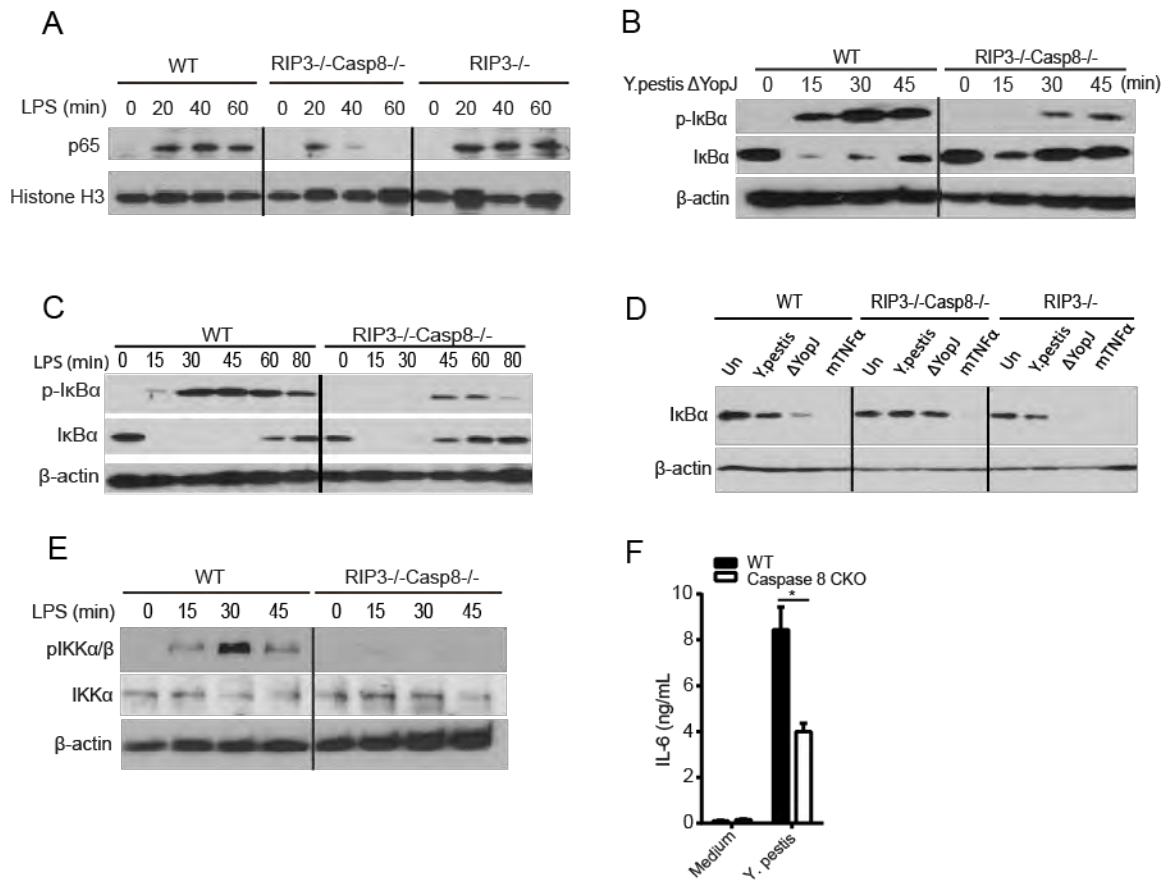
A) Wild type or RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> immortalized mouse macrophages were infected with wild type bacteria or *Y. pestis* ΔYopJ for 2.5 hrs and caspase-3 was determined in cell lysates by Western blot. Cleavage and activation of caspase-3 induced by *Y. pestis* is dependent upon YopJ and absent in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages. B) Wild type or RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> immortalized mouse macrophages were infected with *Y. enterocolitica* (MOI 20) or *Pseudomonas aeruginosa* (MOI 40) for 4 hrs. Cell death was determined by measuring LDH in the supernatant. C) Wild type immortalized macrophages were infected with *Y. pestis* for the indicated time points and gentamycin was added at time points beyond 1 hr. Western blot was used to detect the *Y. pestis*-induced degradation of cIAP1 during infection. Figures are representative for three experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)



**Figure 2.12** The release of cytokines and pro-IL-1 $\beta$  transcription and synthesis induced by *Y. pestis* are impaired in RIP3 $^{-/-}$  caspase-8 $^{-/-}$  macrophages.

A-B) Wild-type (WT) or mutant BMDMs were infected with *Y. pestis*, *Y. pseudotuberculosis* (MOI 10) or treated with LPS (100 ng/mL) or Pam3Cys (500 ng/mL) for 6 hrs, and cytokine release was measured by ELISA. C) Immortalized macrophages were infected with *Y. enterocolitica* (MOI 10) or Sendai Virus (200 pfu) for 20 hrs. TNF $\alpha$  production was detected by ELISA. D) BMDMs were infected with *Y. pestis* for 4 hrs, mRNA was isolated and qPCR for pro-IL-1 $\beta$  was performed. E) BMDMs were infected with *Y. pestis* or *Y. pestis*  $\Delta$ YopJ (MOI 10) for 6 hrs and pro-IL-1 $\beta$  was detected in the cell lysates through Western blot. Figures are representative for 3-8 experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)

not by TNF $\alpha$  in T cells, B cells or NK cells (Su et al., 2005). The cytokine reduction in RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> macrophages suggests that the NF- $\kappa$ B pathway is affected by the deficiency of RIP3 and caspase-8 in response to *Yersinia* or LPS, but not by TLR2 ligands or Sendai virus. To directly evaluate whether NF- $\kappa$ B activation by *Yersinia* is attenuated in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, we detected the different components of NF- $\kappa$ B pathway respectively. Since YopJ can inhibit NF- $\kappa$ B activation (see Chapter I), we stimulated wild type, RIP3<sup>-/-</sup> and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages with the *Y. pestis*  $\Delta$ YopJ mutant strain or LPS for indicated time points as shown in Fig. 2.13. First, p65 nuclear translocation was decreased in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages at 40 and 60 minutes after LPS stimulation (Fig. 2.13A). Next, we detected the phosphorylation of I $\kappa$ B $\alpha$ , which leads to its own degradation and thus releases the p65 to translocate into nucleus. Upon *Y. pestis*  $\Delta$ YopJ or LPS stimulation, the phosphorylation of I $\kappa$ B $\alpha$  was reduced in RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> macrophages compared with wild type cells (Fig. 2.13BC). Accordingly, the degradation of I $\kappa$ B $\alpha$  in DKO macrophages was impaired at later time points (30 or 45 minutes) after adding bacteria or LPS (Fig. 2.13BC). I $\kappa$ B $\alpha$  degradation induced by TNF $\alpha$  stimulation was not affected in DKO cells (Fig. 2.13D), suggesting that the deficiency of caspase-8 and RIP3 does not affect NF- $\kappa$ B activation by all stimuli, which is consistent with a previous report (Su et al., 2005). IKK $\alpha/\beta$  is the upstream kinase responsible for phosphorylating I $\kappa$ B $\alpha$  and phosphorylation of IKK $\alpha/\beta$  leads to its activation. As shown in Fig. 2.13E, the phosphorylation of IKK $\alpha/\beta$  induced by LPS was also suppressed in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, suggesting that the defect is located upstream of IKK $\alpha/\beta$ . The cytokine production, as well as I $\kappa$ B $\alpha$  degradation was intact in RIP3<sup>-/-</sup> cells in contrast to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, suggesting that caspase-8 contributes to NF- $\kappa$ B activation. Similar to DKO cells, IL-6 release induced by *Y. pestis* was significantly decreased in caspase-8 CKO macrophages (Fig. 2.13F), indicating that the attenuation of NF- $\kappa$ B



**Figure 2.13 NF-κB signaling pathway is attenuated in RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> macrophages.**

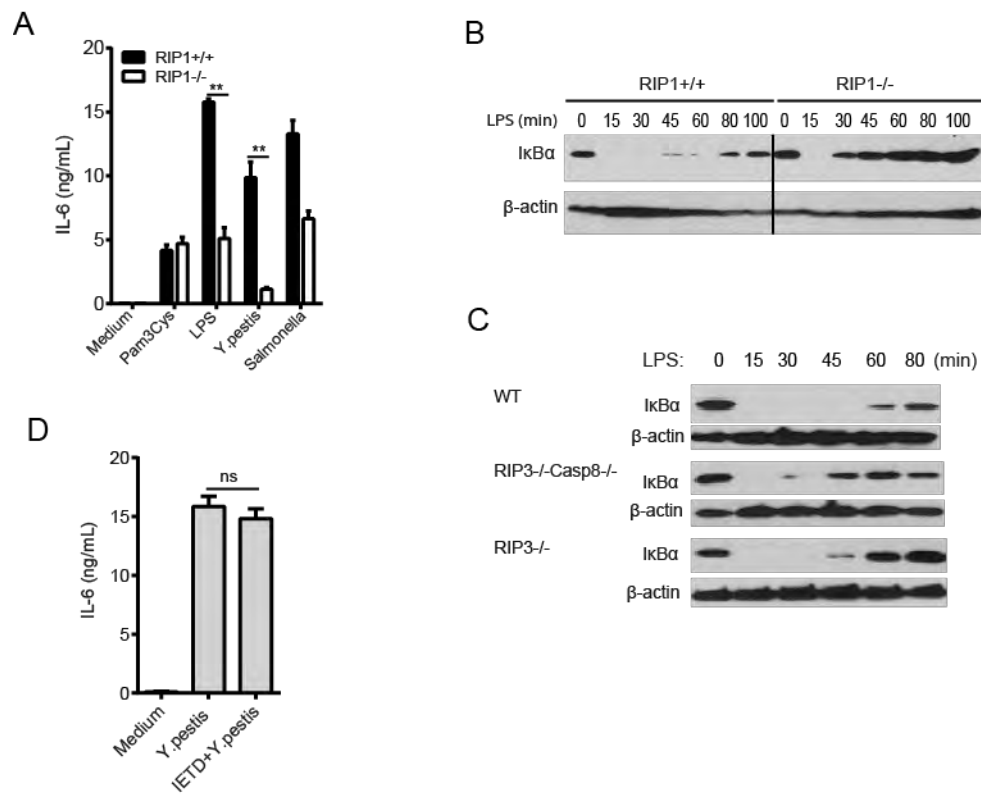
A) Immortalized macrophages were stimulated with LPS (100 ng/mL) for the indicated time points. Nuclear extracts were probed by immunoblot for p65 and Histone-3. B-E) BMDMs were infected with *Y. pestis* ΔYopJ (MOI 10) or treated with LPS (100 ng/mL), mouse TNFα (10ng/mL, 10 min), and cell lysates were probed by immunoblot for the indicated proteins (IκBα, phospho-IκBα, phospho-IKKα/β, IKKα or β-actin). F) Wild type or caspase-8 CKO macrophages were infected with *Y. pestis* (MOI 10) for 6 hours and IL-6 secretion in the supernatant was measured by ELISA. Figures are representative of 2-5 experiments performed. Bars indicate means plus SD. \*p<0.05 (two-tailed t-test)

activation in the DKO cells are due to the deficiency of caspase-8 and not the dual deletion of both RIP3 and caspase-8.

RIP1 is involved in NF- $\kappa$ B activation induced by TNF- $\alpha$ , TLR4 and TLR3 signaling (Cusson-Hermance et al., 2005; Hsu et al., 1996). In our model of *Yersinia* infection, reduced IL-6 production could also be observed in RIP1<sup>-/-</sup> fetal liver macrophages (Fig. 2.14A). In addition, I $\kappa$ B $\alpha$  degradation was attenuated in RIP1-deficient macrophages and showed a similar degradation pattern to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> cells and the I $\kappa$ B $\alpha$  degradation at later time points of 30 and 45 min upon LPS treatment was inhibited (Fig. 2.14BC). How caspase-8 regulates NF- $\kappa$ B activation is currently unclear and may not require the self-processing of caspase-8 (Kang et al., 2008). Our results indicate that the caspase-8 inhibitor IETD did not affect NF- $\kappa$ B-mediated IL-6 production (Fig. 2.14D). However, TRIF mediated pathways may be targeted since MyD88-dependent TLR2 signaling is not affected in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> cells. RIP1 has been suggested to interact with TRIF through their RHIM domains to transduce signaling to activate NF- $\kappa$ B (Cusson-Hermance et al., 2005), and since RIP1<sup>-/-</sup> cells showed a similar I $\kappa$ B $\alpha$  degradation pattern to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> cells that the later time points were influenced, caspase-8 might also affect TRIF-mediated late phase of NF- $\kappa$ B activation.

### **RIP1, caspase-8 and RIP3 mediate inflammasome activation**

In addition to IL-6 and TNF $\alpha$ , we also observed that release of the cytokines IL-1 $\beta$  and IL-18 was significantly inhibited in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages but not in RIP3<sup>-/-</sup> cells when stimulated by *Y. pestis* (Fig. 2.15AB). However, IL-1 $\beta$  release following stimulation with Pam3Cys and nigericin was intact in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.15A), implying that not all universal IL-1 $\beta$  production machinery were affected, like the canonical NLRP3 activation herein

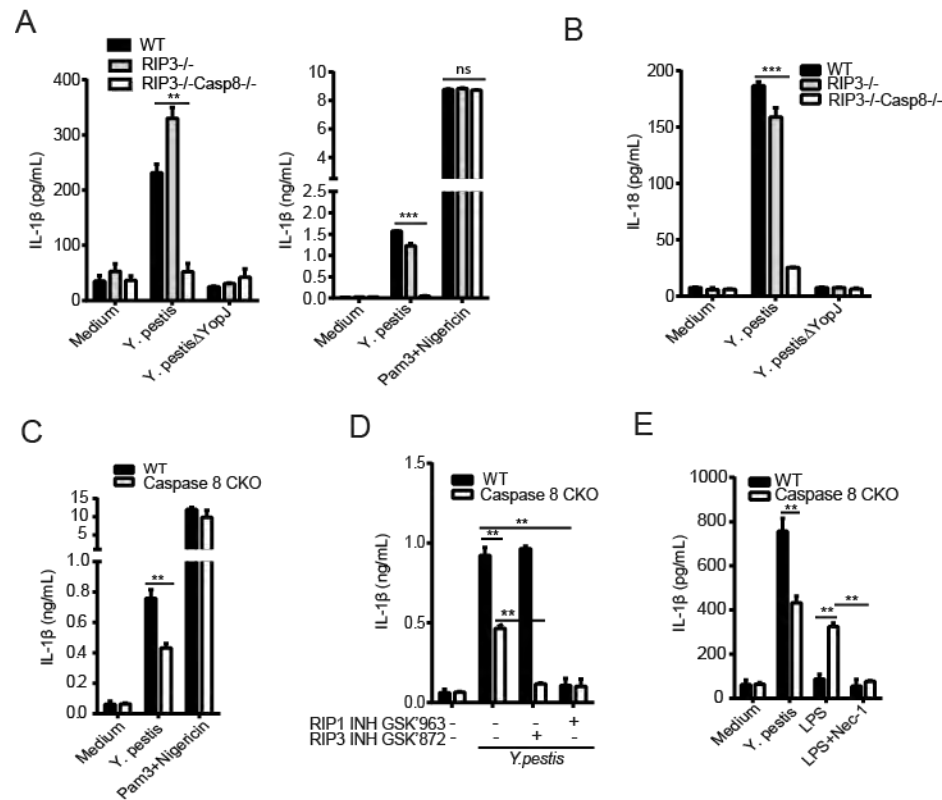


**Figure 2.14 RIP1 also contributes to cytokine release and NF-κB activation induced by *Y. pestis* or LPS.**

A) WT or RIP1<sup>-/-</sup> fetal liver macrophages were stimulated with LPS (50 ng/mL), Pam3Cys (500 ng/mL), *Y. pestis* (MOI 10) or *Salmonella* (MOI 1.5) for 6 hrs. IL-6 release was measured by ELISA. B-C) Fetal liver macrophages (B) or immortalized macrophages (C) were stimulated with LPS (100 ng/mL) for the indicated time points. The IκBα degradation in cell lysates was detected by Western blot. C) In RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> macrophages, the IκBα degradation was affected at 30 min and 45 min, but not at early 15 min time point, when compared with WT macrophages. D) Caspase-8 enzymatic inhibitor IETD (40 μM) doesn't affect *Y. pestis*-induced IL-6 production. Figures are representative for three experiments performed. Bars indicate mean plus SD. \*\*p<0.01 (two-tailed t-test)

triggered by Pam3Cys and nigericin. As suggested previously (Lilo et al., 2008; Vladimer et al., 2012), *Y. pestis*-induced IL-1 $\beta$  and IL-18 production is dependent on YopJ, since *Y. pestis*  $\Delta$ YopJ mutant infection did not trigger any detectable IL-1 $\beta$  secretion (Fig. 2.15A,B). IL-1 $\beta$  production was partially decreased in caspase-8 CKO macrophages, in contrast to complete inhibition in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> DKO cells (Fig. 2.15C). RIP3 inhibition by the kinase inhibitor GSK'862 could further inhibit the residual IL-1 $\beta$  secretion in caspase-8 CKO cells (Fig. 2.15D). RIP1 kinase inhibitor GSK'963 pretreatment completely blocked IL-1 $\beta$  release induced by *Y. pestis* (Fig. 2.15D). While the absence of caspase-8 alone in macrophages decreased IL-1 $\beta$  release induced by *Yersinia* infection, it prompted IL-1 $\beta$  production by LPS treatment (Fig. 2.15E), similarly to that suggested for caspase-8 CKO dendritic cells in which LPS alone can induce IL-1 $\beta$  production (Kang et al., 2013). This IL-1 $\beta$  release in caspase-8 CKO cells by LPS could be blocked by the RIP1 inhibitor Necrostatin-1, indicating that it is RIP1 kinase-mediated and shows consistency with studies by Kang et al. (2013) (Fig. 2.15E). Thus, more intricate stimulations, like those observed during infections, yield a different result than a purified ligand like LPS, possibly reflecting combined effects induced by both LPS and the *Yersinia* T3SS effector YopJ in the context of live bacteria.

Our previous data (Fig. 2.12, 2.13) could partially explain reduced IL-1 $\beta$  release, due to the impairment of NF- $\kappa$ B activation in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, which contributes to the transcription and synthesis of pro-IL-1 $\beta$ . Unlike IL-6 and TNF $\alpha$ , the release of mature IL-1 $\beta$  and IL-18 requires additional stimuli to trigger pro-IL-1 $\beta$  processing into mature cytokines, normally mediated by active caspase-1. Although, caspase-8 is also suggested to be able to directly cleave pro-IL-1 $\beta$  independent of caspase-1 when stimulated with TLR ligands poly (I:C) or LPS (Maelfait et al., 2008), Fas ligand (Bossaller et al., 2012), or fungi and mycobacteria sensed by

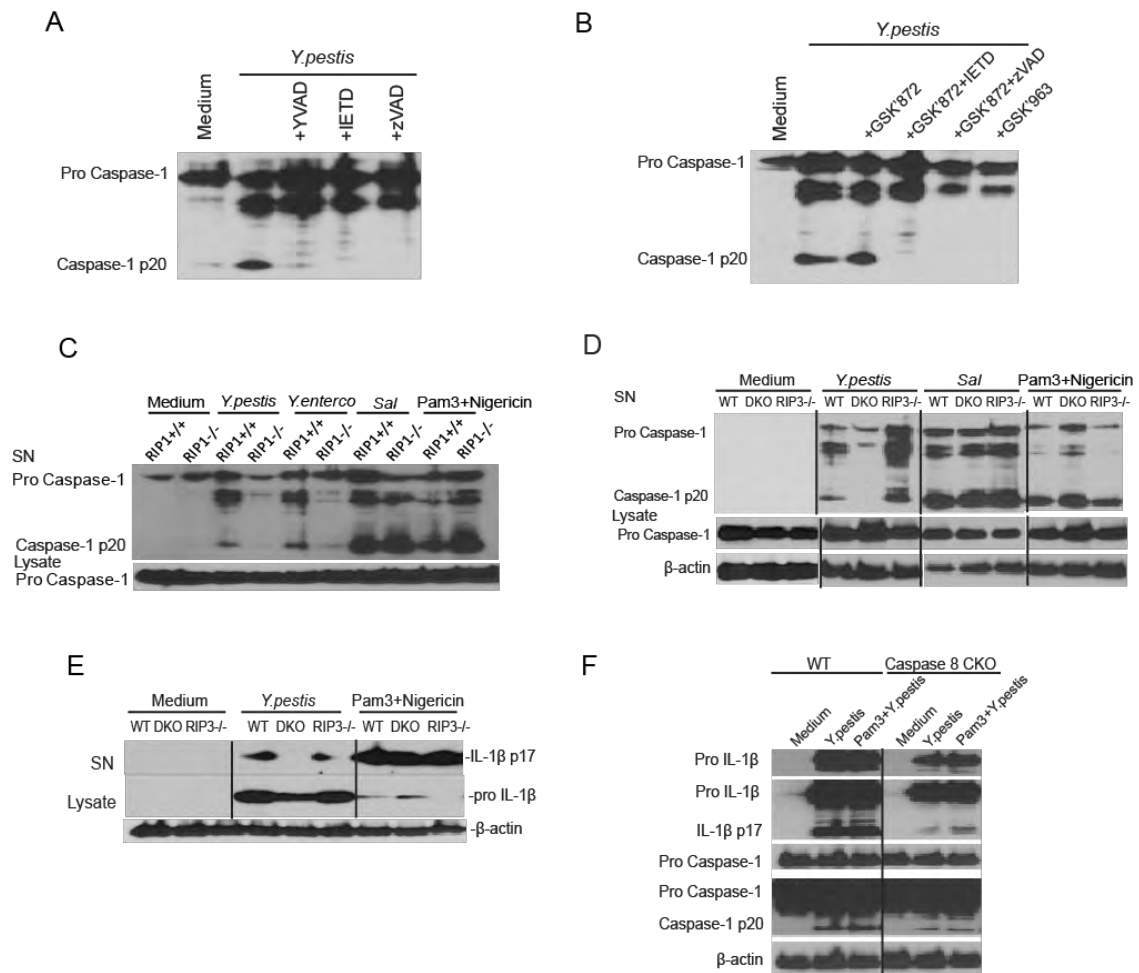


**Figure 2.15** *Y. pestis*-induced release of IL-1 $\beta$  and IL-18 is severely reduced in RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> macrophages.

A-E) BMDMs were infected with *Y. pestis* or *Y. pestis*  $\Delta$ YopJ (MOI 10) for 6 hrs, or stimulated with nigericin (10 $\mu$ g/ml) for 1 hr after priming with Pam3Cys (4 hrs, 500 ng/ml). IL-1 $\beta$  and IL-18 were analyzed by ELISA. D) Some BMDMs were treated with RIP1 inhibitor GSK'963 (1  $\mu$ M) or RIP3 inhibitor GSK'872 (10  $\mu$ M) for 1hr before infection. E) BMDMs were challenged with *Y. pestis* (MOI 10) for 6 hrs or LPS (50 ng/mL) for 10 hrs with or without Nec-1 pretreatment (20  $\mu$ M). Figures are representative of 3-5 experiments. Bars indicate means plus SD. \*\*p<0.01, \*p<0.05 (two-tailed t-test in A,B,C, and two-way ANOVA with Tukey's post-test in D,E).



dectin-1 (Gringhuis et al., 2012). Surprisingly, caspase-8 or RIP1 inhibition minimized *Y. pestis*-induced caspase-1 cleavage (Fig. 2.16AB), implying direct effects on inflammasome action by caspase-8 and RIP1. To address this question, we infected wild type, RIP3<sup>-/-</sup> and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, or RIP1<sup>+/+</sup> and RIP1<sup>-/-</sup> fetal liver macrophages with *Y. pestis* and assessed caspase-1 processing. As shown in Fig. 2.16CD, caspase-1 cleavage induced by *Yersinia* was inhibited in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> and RIP1<sup>-/-</sup> macrophages, but not in RIP3-deficient cells. Caspase-1 cleavage induced by *Salmonella* and Pam3Cys plus nigericin stimulation was not affected (Fig. 2.16CD), indicating that NLRC4- and NLRP3-mediated caspase-1 cleavage is not inherently reduced in RIP1<sup>-/-</sup> or DKO cells. Moreover, the pro-caspase-1 expression level in cell lysates was not obviously changed in RIP1<sup>-/-</sup> or RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> cells (Fig. 2.16CD), suggesting the deficiency of RIP1 or caspase-8/RIP3 affects the processing of pro-caspase-1 into its active, cleaved form. Consistent with the ELISA results of IL-1 $\beta$  release, Western blot also demonstrates that the secretion of mature IL-1 $\beta$  into cell supernatant by *Y. pestis* was inhibited in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.16E). In cell lysates of caspase-8 CKO macrophages, both cleaved caspase-1 and mature IL-1 $\beta$  production were attenuated compared with wild type cells (Fig. 2.16F), further suggesting the involvement of caspase-8 in caspase-1 cleavage when infected with *Y. pestis*. Although we cannot exclude the possibility that caspase-8 directly cleaves pro-IL-1 $\beta$  during *Y. pestis* challenge, our western blot results (Fig. 2.16) propose another effect of caspase-8 in inflammation, directly inducing caspase-1 cleavage and activation, in a RIP3-enhanced manner. To further investigate the underlying mechanism, we first checked the potential upstream players which might be involved in caspase-8 activation and the related IL-1 $\beta$  production. As shown in Fig. 2.17ABC, both IL-1 $\beta$  release and caspase-8 activity induced by *Y. pestis* was not significantly affected in cells deficient in TNF $\alpha$ , IFN $\alpha$ R or FasL, suggesting that caspase-8 activation and IL-1 $\beta$  maturation appear independent of TNF $\alpha$ , type I IFN and FasL and



**Figure 2.16** RIP kinases and caspase-8 control caspase-1 cleavage induced by *Y. pestis*.

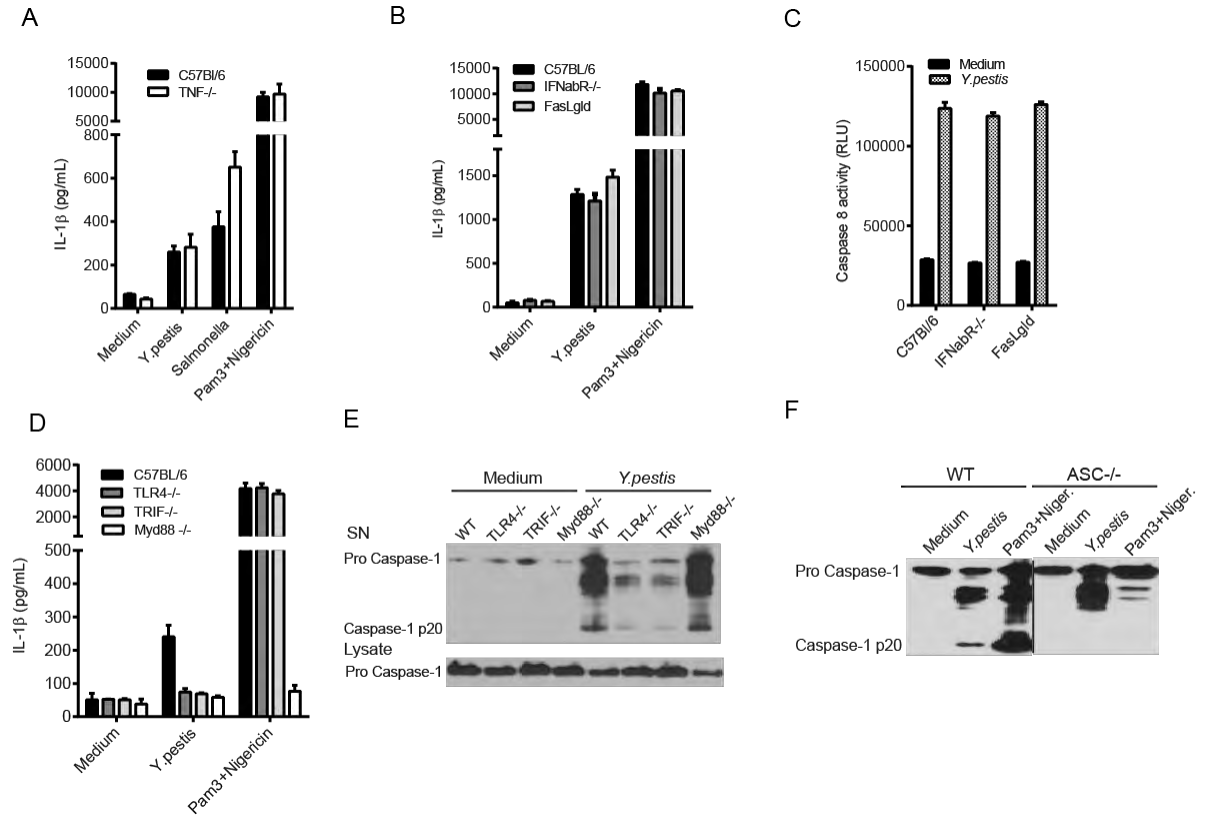
A-B) Wild type BMDMs were pretreated with inhibitors (caspase-1 inhibitor YVAD, 20  $\mu$ M; caspase-8 inhibitor IETD, 40  $\mu$ M; pan-caspase inhibitor zVAD, 20  $\mu$ M; RIP1 kinase inhibitor GSK'963, 1  $\mu$ M; RIP3 kinase inhibitor GSK'872, 10  $\mu$ M) for 1 hr before *Y. pestis* infection (MOI 10, 6 hrs). The supernatants were harvested and assayed for caspase-1 processing. C-F) BMDM (WT, RIP3<sup>-/-</sup>, RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> DKO, caspase-8 CKO) or fetal liver macrophages (RIP1<sup>+/+</sup>, RIP1<sup>-/-</sup>) were infected with *Y. pestis*, *Y. enterocolitica* (MOI 10) or *Salmonella* (Sal, MOI 1.5) for 6 hrs or primed with Pam3Cys followed by nigericin or *Y. pestis* for 1 hr or 6 hrs, and supernatants (SN) or lysates (F) were analyzed for caspase-1 or IL-1 $\beta$  processing by immunoblots. Figures are representative of three experiments.

may have some common features with responses induced by certain drugs or *Citrobacter* (Antonopoulos et al., 2013; Gurung et al., 2014; Shenderov et al., 2014). However, both caspase-1 cleavage and IL-1 $\beta$  production stimulated by *Y. pestis* were obviously reduced in TLR4<sup>-/-</sup> and TRIF<sup>-/-</sup> macrophages (Fig. 2.17DE). In MyD88<sup>-/-</sup> macrophages, the caspase-1 processing was similar to wild type cells but the IL-1 $\beta$  release was completely abrogated, which can be attributed to MyD88's critical role in the NF- $\kappa$ B activation pathway to induce the transcription and synthesis of pro-IL-1 $\beta$  (Fig. 2.17DE). Similar to the cell death results (Fig. 2.8BC), caspase-1 processing in TLR4<sup>-/-</sup> and TRIF<sup>-/-</sup> cells was partially decreased unlike the complete inhibition observed in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, suggesting the pathways to regulate caspase-1 cleavage and induce cell death have common players. TLR4-TRIF signaling is not necessarily required, but contributes to amplify the effects.

It is unclear how caspase-8 executes the cleavage of caspase-1. Our results suggest that the inflammasome adaptor ASC might play a role as the caspase-1 cleavage induced by *Y. pestis* was inhibited in ASC-deficient macrophages (Fig. 2.17F). ASC can associate with caspase-8 after *Francisella* or *Salmonella* infection (Man et al., 2013; Pierini et al., 2012), although the role of ASC may differ depending upon conditions and source of YopJ (Brodsky et al., 2010; Vladimer et al., 2012; Zheng et al., 2011).

## **Role of caspase-8 and RIP3 for *in vivo* resistance to bacterial infection**

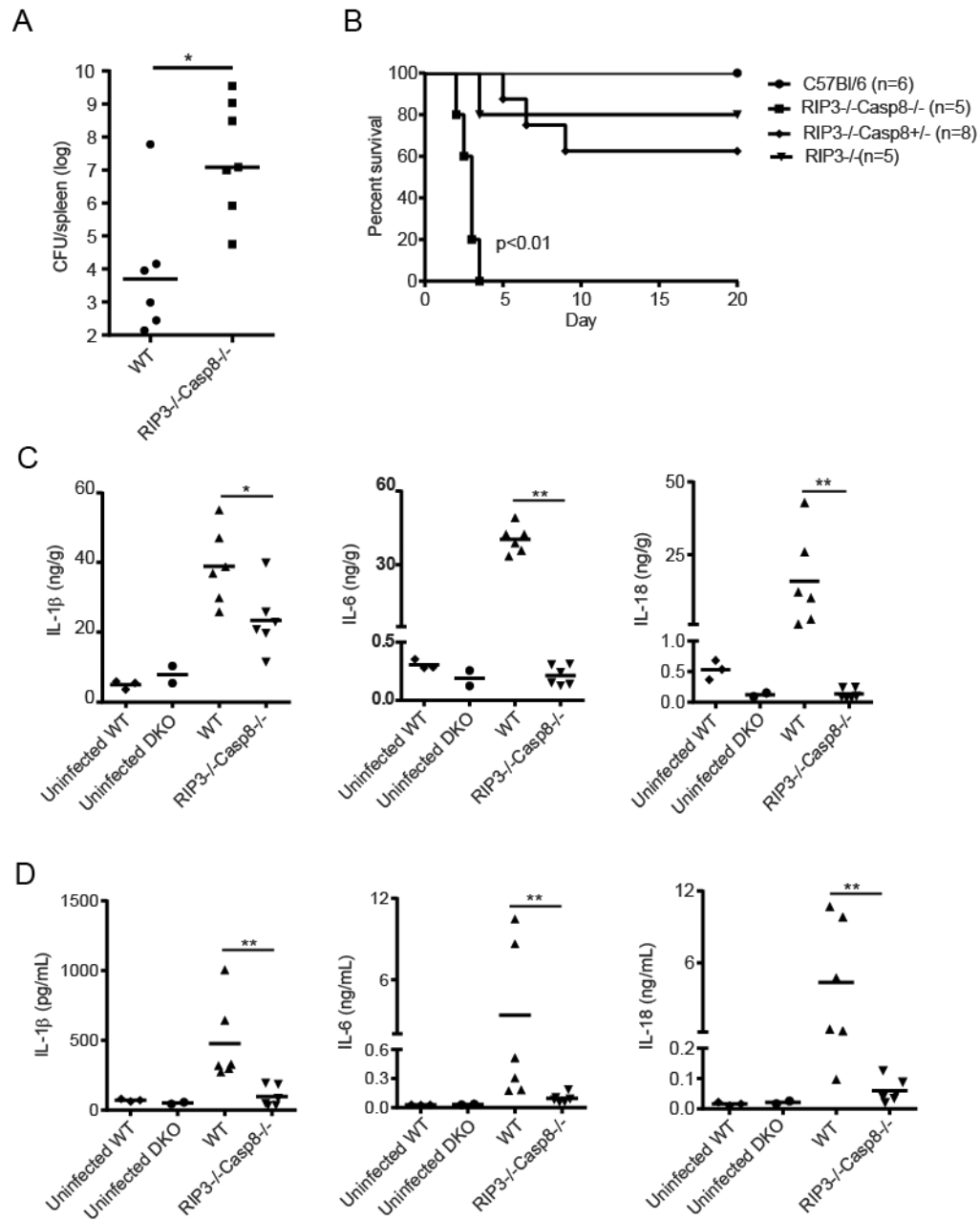
The results described above suggest that caspase-8 and RIP kinases play critical roles in the regulation of *Y. pestis* induced cell death, inflammasome activation and cytokine release *in vitro*. The *in vivo* relevance of these findings was therefore addressed in a murine model of plague.



**Figure 2.17** *Y. pestis*-induced IL-1 $\beta$  release or caspase-1 cleavage are reduced in TLR4-, TRIF- and ASC-deficient BMDMs, but not in MyD88<sup>-/-</sup>, TNF<sup>-/-</sup>, IFN $\alpha$  $\beta$ R<sup>-/-</sup> and FasL<sup>gld</sup> BMDMs.

A,B,D) BMDMs from TNF<sup>-/-</sup>, IFN $\alpha$  $\beta$ R<sup>-/-</sup>, FasL<sup>gld</sup>, TLR4<sup>-/-</sup>, TRIF<sup>-/-</sup> and MyD88<sup>-/-</sup> mice were stimulated with *Y. pestis* (MOI 10) for 6 hours, then the IL-1 $\beta$  in supernatant was detected via ELISA. C) Caspase-8 activity induced by *Y. pestis* is not dependent on IFN $\alpha$  $\beta$ R1 and FasL. Caspase-8 activation was detected in C57BL/6, IFN $\alpha$  $\beta$ R1<sup>-/-</sup> and FasL<sup>gld</sup> BMDMs which were infected with 40 MOI *Y. pestis* for 2 hrs, using Caspase-Glo 8 assay. E-F) BMDMs were infected with *Y. pestis* (MOI 10) for 6 hrs. Then the supernatants (SN) or lysates were analyzed for caspase-1 processing by immunoblots. Figures are representative of three experiments.

First, we infected wild type C57Bl/6 and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice with fully virulent *Y. pestis* KIM1001 (300 cfu, s.c.). Sixty-eight hours later, spleens were harvested and homogenized for analysis of bacterial load. As demonstrated in Fig. 2.18A, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> DKO mice have significantly higher numbers of bacteria in their spleens compared to wild type mice, strongly suggesting that DKO mice were more susceptible to subcutaneous infection with virulent *Y. pestis*. The LD50 of fully virulent KIM1001 via subcutaneous route in mice is very low, less than 10 cfu (Sodeinde et al., 1992), making it very difficult to control the infection and monitor the increased susceptibility in genetically modified mice. To overcome this disadvantage, we used the attenuated strains KIM1001-EcLpxL or KIM1001-YtbLpxL, which express *E.coli* lpxL or *Y. pseudotuberculosis* lpxL, respectively. These strains constitutively synthesize a TLR4-activating hexa-acylated LPS even at 37°C. The LD50 of these attenuated strains is greater than 1x10<sup>7</sup> (Montminy et al., 2006). We have successfully utilized the attenuated *Y. pestis* strains to investigate the contribution of various innate immune components, such as TLR4, MyD88, NLRP12, IL-1 $\beta$  or IL-18, to the innate defensive response against bacterial infection (Montminy et al., 2006; Vladimer et al., 2012). In addition, the *in vitro* results indicated that the modified strain *Y. pestis*-EcLpxL induced caspase-8/RIP3-dependent cell death, similarly to wild type *Y. pestis* (Fig. 2.9A). Hence, to determine whether the deficiency in RIP3 and caspase-8 affect the survival outcome of *Y. pestis* infection, we infected the C57Bl/6, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup>, RIP3<sup>-/-</sup> and RIP3<sup>-/-</sup>caspase-8<sup>+/-</sup> mice subcutaneously with 500 cfu of KIM1001-EcLpxL and monitored the mice for survival. As shown in Fig. 2.18B, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice were significantly more sensitive to infection, with all mice succumbing to disease before day 5. To analyze whether the deficiency of RIP3 and caspase-8 also affect the cytokine production *in vivo*, the mice were infected intravenously with 500 cfu KIM1001-EcLpxL for 42 hours, and the spleen homogenates and serum were collected for cytokine measurement by ELISA. Compared with uninfected controls, *Y. pestis* challenge induced obvious production of IL-1 $\beta$ , IL-18 and IL-6 in both spleen



**Figure 2.18 Caspase-8 with RIP3 is critical for *in vivo* resistance to bacterial infection.**

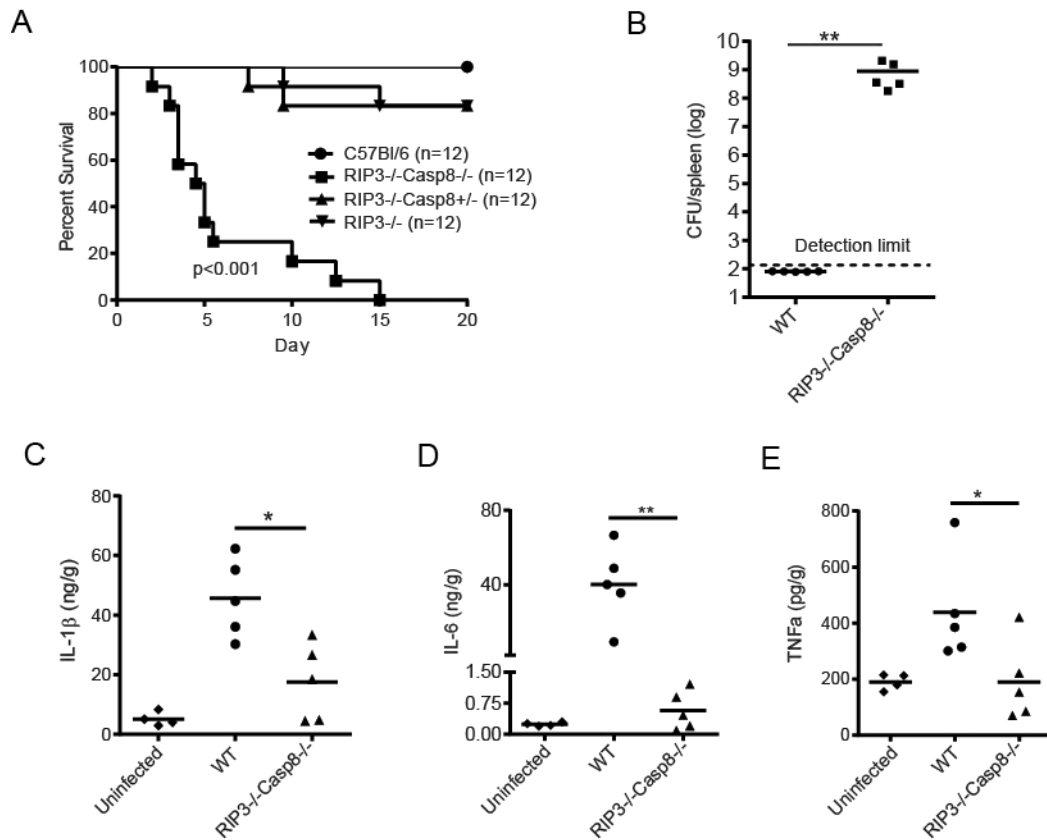
A) RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> DKO or WT mice were infected s.c. with virulent *Y. pestis* KIM1001 (300 CFU) for 68 hrs and spleens were analyzed for bacterial growth. B) C57Bl/6, RIP3<sup>-/-</sup>, RIP3<sup>-/-</sup>Casp8<sup>+/-</sup> or RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice were infected subcutaneously with an attenuated strain of *Y. pestis*, KIM1001-EcLpxL (500 cfu) and monitored for survival. RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice were significantly more sensitive to infection and all died before day 5. EcLpxL-expressing *Y. pestis* is an attenuated

strain of KIM1001 that has TLR4-activating properties and utilizes the same RIP3/Caspase-8-dependent cell death pathway (Fig. 2.9A) making this an ideal strain for survival studies. C-D) Wild type or RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice were uninfected or infected intravenously (i.v.) with 500 cfu of KIM1001-EcLpxL and spleen homogenates (C) and serum (D) were collected for IL-1 $\beta$ , IL-6 and IL-18 measurement by ELISA. In the spleen (C) cytokine levels are displayed as ng cytokine per g of tissue. IL-1 $\beta$ , IL-6 and IL-18 were significantly reduced in RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice in both the spleen and serum compared to wild type animals. Shown is a representative experiment out of 2-3 performed. \*p<0.05, \*\*p<0.01 (Mann-Whitney U test).

and serum, while all the cytokine levels were significantly decreased in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> DKO mice (Fig. 2.18CD), showing consistency with the *in vitro* results.

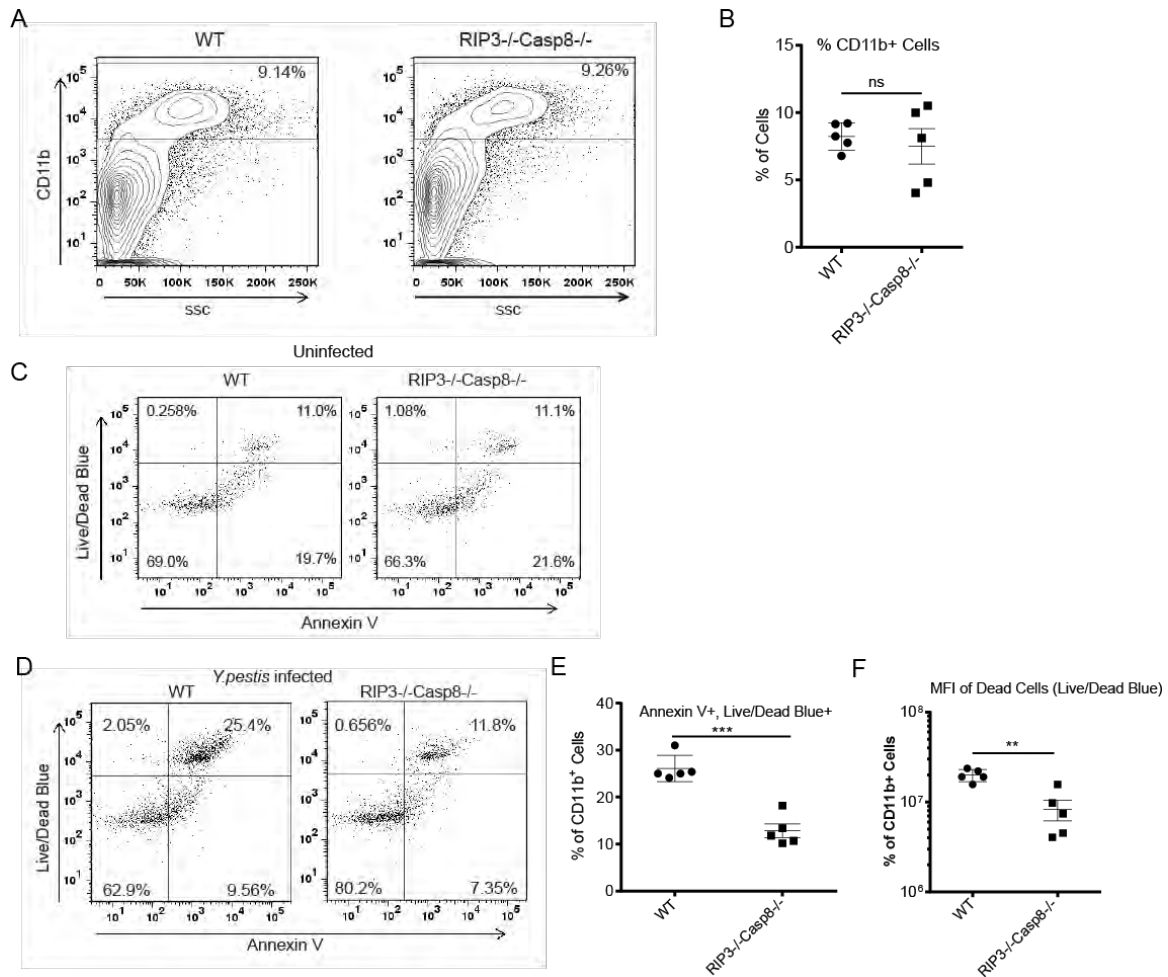
To investigate whether the sensitivity of RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice is due to the hematopoietic originating cells, WT mice were lethally irradiated (900 rads) and received bone marrow transplantation (BMT) from either WT, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup>, RIP3<sup>-/-</sup> or RIP3<sup>-/-</sup>caspase-8<sup>+/-</sup> mice. Six weeks after bone marrow transplantation, mice were infected s.c. with 500 cfu of *Y. pestis* KIM1001-EcLpxL and monitored for survival. Similar to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice, all of the BMT mice reconstituted with RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> bone marrow cells succumbed to infection, with a few DKO mice that progressed slower and died after 5 days of infection (Fig. 2.19A). Moribund mice had large numbers of bacteria in their spleens compared to WT controls, suggesting that death occurred from uncontrolled systemic bacterial replication (Fig. 2.19B). This correlated with decreased IL-1 $\beta$ , TNF $\alpha$  and IL-6 cytokine levels in spleen homogenates (Fig. 2.19CDE), phenocopying the regular RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> DKO mice (Fig. 2.18B-D). In addition, single-cell suspensions of splenocytes from i.v. infected BMT mice (500 cfu, 42 hours) were stained with CD11b-PE antibody to identify myeloid cells and with live/dead blue and annexin V-FITC to determine cell viability. As indicated in Fig. 2.20AB, single splenocytes were gated for CD11b expression and there was no significant difference in the percentage of CD11b<sup>+</sup> cells between WT and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMT mice. Of the CD11b<sup>+</sup> cells, there was no difference in the frequency of dead cells (live/dead blue<sup>+</sup>, annexin V<sup>+</sup>) in the uninfected WT and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice (Fig. 2.20C). However, when subjected to *Y. pestis* infection, the percentage of dead cells (live/dead blue<sup>+</sup>, annexin V<sup>+</sup>) in CD11b-positive myeloid splenocytes was significantly lower in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMT mice than WT controls (Fig. 2.20DE). Furthermore, the mean





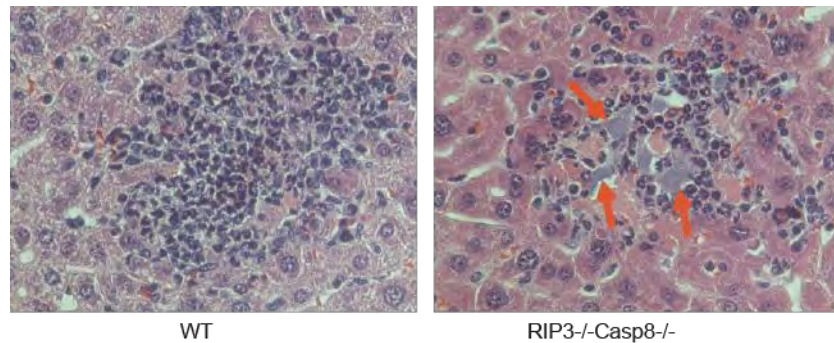
**Figure 2.19 Deficiency of caspase-8 and RIP3 in hematopoietic compartment makes mice susceptible to *Y. pestis* infection.**

A-B) Lethally irradiated mice, subjected to bone marrow transplantation (BMT) from the indicated genotypes, were infected s.c. with 500 CFU of *Y. pestis* KIM1001-EcLpxL and monitored for survival (A), p < 0.001 DKO vs WT (log-rank test). Spleens from moribund DKO BMT mice and live WT controls were analyzed for bacterial loads (B). C-E) Mice from BMT as above were infected i.v. with KIM1001-EcLpxL (500 CFU) for 42 hrs. Spleens were homogenized and analyzed for cytokines by ELISA [as cytokine/g tissue]. Shown is a representative experiment out of 2-3 performed. \*p < 0.05, \*\*p < 0.01 (Mann-Whitney U test)



**Figure 2.20** *Y. pestis* infection induces more death of CD11b-positive myeloid cells in WT spleens than RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> spleens.

A-F) Lethally irradiated mice, transplanted with bone marrow cells (BMT) from wild type or RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice were infected i.v. with 500 CFU of *Y. pestis* KIM1001-EcLpxL for 42 hours. Single-cell suspensions were made from spleens and stained with CD11b-PE to identify myeloid cells and with live/dead blue and Annexin V-FITC to determine cell viability. A) Single cells were gated for CD11b expression, and there were no significant differences in the percentage of CD11b<sup>+</sup> cells in wild type or RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> BMT mice (B). C) Of CD11b<sup>+</sup> cells, there was no difference in the frequency of dead cells (Live/Dead blue<sup>+</sup>, Annexin V<sup>+</sup>) in the uninfected WT and RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> BMT mice. D-E) *Y. pestis* infection induced significantly more dead CD11b<sup>+</sup> cells in WT than RIP3<sup>-/-</sup>caspase8<sup>-/-</sup> BMT mice. F) The mean fluorescent intensity (MFI) of live/dead blue stain in CD11b<sup>+</sup> cells was significantly higher in wild type compared to RIP3<sup>-/-</sup>Casp8<sup>-/-</sup> mice. Shown is a representative experiment out of two performed. \*\*p<0.01 (Mann-Whitney U test)



**Figure 2.21 Liver histology suggests less controlled infection in RIP3<sup>-/-</sup> caspase-8<sup>-/-</sup> BMT mice.**

Lethally irradiated mice, transplanted with bone marrow cells (BMT) from wild type or RIP3<sup>-/-</sup> Casp8<sup>-/-</sup> mice, were infected i.v. with 500 CFU of *Y. pestis* KIM1001-EcLpxL for 42 hours. Livers were harvested, fixed and stained with hematoxylin and eosin and subjected to microscopy (400x). Foci containing inflammatory cells (mostly neutrophils) are shown, with visible pockets containing bacteria indicated by arrows. Shown is a representative experiment out of two performed.

fluorescent intensity (MFI) of live/dead blue stain in CD11b<sup>+</sup> cells was also significantly higher in WT compared with RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMT mice (Fig. 2.20F), suggesting that *Y. pestis* infection induced more myeloid cell death in WT spleen than RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> spleen, in agreement with *in vitro* studies. We also harvested the livers of BMT mice 42 hours after i.v. infection with KIM1001-EcLpxL. The liver sections were stained with hematoxylin and eosin. As shown in Fig. 2.21, there were visible bacteria-containing pockets in inflammatory foci in the livers of DKO BMT mice but not present in infected WT control mice, suggesting that RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMT mice failed to suppress bacterial growth *in vivo*.

Since irradiated mice that received RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMT behaved similarly as regular DKO animals, we propose that protection towards infection is mediated by cells expressing caspase-8 and RIP3 that originate from the bone marrow. Some questions still remain with respect to certain details of how caspase-8 and RIP3 are involved in caspase-1 processing, although it is possible that ASC has a central role. Our results provide a basis for increased understanding of how bacterial pathogens, via their T3SS, interact with several aspects of host innate immunity through RIP kinases and caspase-8. The data also show how apoptosis, generally viewed as a “silent” cell death, can be accompanied by strong inflammatory reactions, via pathways with several common players. The host may have developed these pathways as an effective means of alerting cells to the infection. We propose that caspase-8 and RIP kinases are central regulators of cell death and innate immune responses to *Y. pestis*, and we establish a role for these components in anti-bacterial innate immune responses. Therapies that modulate the activity of these pathways may be useful in the treatment of bacterial infections.

## **CHAPTER III Discussion**

This thesis research has focused on the mechanisms of *Y. pestis*-induced cell death and innate immune responses. We demonstrate that RIP1, caspase-8 and RIP3 are critical host factors to control cell fate upon *Yersinia* infection. RIP1 and caspase-8 also play important roles to regulate innate immune responses against *Yersinia* stimulation, including NF- $\kappa$ B and caspase-1 activation and thereby the release of IL-6, TNF $\alpha$ , IL-1 $\beta$  and IL-18 cytokines. Consistent with our *in vitro* results, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> (DKO) mice produce significantly lower levels of IL-6, IL-1 $\beta$  and IL-18 in the serum and spleen upon infection. Infected RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice also have decreased myeloid cell death in the spleen compared with WT mice. Consequently, DKO mice are more sensitive to *Y. pestis* infection and all animals succumb quickly upon bacterial challenge with an attenuated *Y. pestis* strain. Both *in vitro* and *in vivo* work suggest that caspase-8 and RIP kinases critically contribute to innate immune responses and cell death elicited by *Y. pestis*, thus providing protection against bacterial infection. Concurrently, Philip et al. published similar results indicating that RIP1, caspase-8 and FADD are required for *Y. pseudotuberculosis* YopJ-induced cell death and caspase-1 activation (Philip et al., 2014), further corroborating the novel and central role of RIP1 and caspase-8 in innate immunity to control *Yersinia* infections.

## Cell death

More than 10 years ago, *Yersinia* bacteria were found to induce robust and rapid cell death in macrophages as well as dendritic cells (DCs) through its T3SS effector YopJ/YopP (Mills et al., 1997; Monack et al., 1997). These studies indicated that *Yersinia*-induced cell death mostly display apoptosis characteristics, although one subsequent study also observed simultaneous necrosis-like death morphology in DCs (Gröbner et al., 2006). Through an electron microscopy approach, here we demonstrate that macrophages infected with *Y. pestis* showed typical apoptotic

morphologies, with nuclear condensation and cell membrane blebbing (Fig. 2.4C). The DNA fragmentation pattern (Fig. 2.4D, 2.6B), which represents the hallmark of apoptosis, further confirms that *Y. pestis* induces apoptosis in wild type macrophages.

RIP1, as a serine/threonine kinase, has both scaffolding (kinase-independent) and non-scaffolding (kinase-dependent) roles, to determine the cell fate when stimulated by death receptor ligation, TLR ligands, RIG-I like receptor activation or cIAPs depletion (Christofferson et al., 2014). Here we expanded the scope by showing that RIP1 also contributes to the regulation of cell fate following bacterial infection. RIP1<sup>-/-</sup> fetal liver macrophages are resistant to cell death induced by *Yersinia* infection (Fig. 2.6A and Philip et al., 2014). RIP1 kinase inhibitors, including the novel inhibitor GSK'963 (Fig. 2.6C) and necrostatin-1 (Philip et al., 2014), efficiently block the *Yersinia*-induced cytotoxicity. Importantly, we have also excluded the possibility that RIP1-mediated cell death by *Y. pestis* is a mere secondary cytotoxicity effect induced by TNF $\alpha$  or Fas ligand, as TNF $\alpha$  or FasL deficient cells are sensitive to *Y. pestis*-induced cell death (Fig. 2.3BC).

For the mechanism of how RIP1 is targeted by *Yersinia* YopJ to induce cell death, we currently have some clues. As described in Chapter I, the modification of RIP1 determines the choice of three distinct cellular fates downstream: NF- $\kappa$ B activation, apoptosis, or necrosis. When RIP1 is highly ubiquitinated, NF- $\kappa$ B will be activated to promote gene transcription and cell survival. When RIP1 is deubiquitinated, either apoptotic or necrotic cell death pathways will be initiated. cIAP1 and cIAP2 are E3 ubiquitin ligases that mediate the Lys63-polyubiquitination of RIP1 (Bertrand et al., 2008). The ubiquitin chains of RIP1 function as docking sites, recruiting downstream molecules including TAK1 to activate the NF- $\kappa$ B pathway. TAK1 also prevents RIP1 to interact with caspase-8 to trigger apoptosis (Bertrand et al., 2008). Two studies have shown that TAK1 can be directly acetylated by *Y. pestis* YopJ (Paquette et al., 2012) or *Y.*

*pseudotuberculosis* YopJ (Meinzer et al., 2012). The acetylation of TAK1 may result in its inhibition, leading to the interaction between RIP1 and caspase-8 and the initiation of apoptosis.

Moreover, the depletion of cIAPs promotes the formation of ripoptosome (also called complex IIa), a signaling platform composed of RIP1, caspase-8 and FADD that leads to caspase-8-mediated apoptosis as well as caspase-independent RIP3-mediated necrosis (Tenev et al., 2011; Feoktistova et al., 2011). The ripoptosome assembly requires RIP1 kinase activity (Tenev et al., 2011). We found that *Yersinia* infection induced cIAP1 depletion in a time-dependent manner (Fig. 2.11C) and cIAP1 degradation requires YopJ (data not shown). Our preliminary data also indicates that YopJ may reduce the ubiquitination of RIP1 (data not shown), suggesting that *Yersinia* YopJ may inhibit the ubiquitination of RIP1 through depleting cIAPs, thus leading to the interaction of RIP1 and caspase-8 (Fig. 2.6D) thereby causing apoptosis. An alternative mechanism could be that YopJ directly acetylates RIP1, through its acetyltransferase activity (as described in Chapter I), and thereby inhibits the phosphorylation and ubiquitination of RIP1. Actually our preliminary data suggests that the phosphorylation of RIP1 is inhibited by YopJ (data not shown), supporting the proposal. Both hypotheses converge on the inhibited ubiquitination of RIP1, which favors the formation of ripoptosome to mediate cell death pathways. How *Yersinia* YopJ targets RIP1, directly or indirectly, is very interesting and needs more work to elucidate the exact mechanism.

*Y. pestis* infection induced the interaction between RIP1 and caspase-8 (Fig. 2.6D), suggesting the formation of ripoptosome during bacterial stimulation. RIP1, caspase-8 and FADD are the core components of ripoptosome, whose formation facilitates cell death by caspase-8-mediated apoptosis or RIP3-mediated necroptosis when caspase-8 is inhibited. Similar to RIP1<sup>-/-</sup> cells, RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> and RIP3<sup>-/-</sup>FADD<sup>-/-</sup> macrophages are completely resistant to *Yersinia*-induced



cytotoxicity (Fig. 2.4, Philip et al., 2014). In the extrinsic apoptosis pathway, caspase-8 acts as the critical initiator caspase which can be activated by death receptor ligation (as described in Chapter I). The caspase-8 activation is induced by its homo-dimerization. The auto-proteolytic cleavage, which separates the N-terminal DED domain from its C-terminal domain, can stabilize the active caspase-8 (Fuentes-Prior and Salvesen, 2004; van Raam and Salvesen, 2012). Cleaved, active caspase-8 is comprised of the large and small subunits along with its catalytic site, to target its substrates including caspase-3 and caspase-7 to trigger apoptosis. During *Y. pestis* infection, caspase-8 is cleaved into 18 kDa subunit and this cleavage depends on the presence of YopJ (Fig. 2.5A). A caspase-8 enzyme assay is a reliable method to quantitatively measure the catalytic activity of caspase-8, and the specificity of this assay has been confirmed using RIP3<sup>-/-</sup>caspase-8<sup>+/-</sup> and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> cells (Fig. 2.5C). Consistently, the caspase-8 enzymatic assay also indicated that wild type *Y. pestis* induced robust caspase-8 activation at 2 hrs post infection while the YopJ mutant strain did not (Fig. 2.5B). Downstream of caspase-8, caspase-3 and -7 are substrates of caspase-8 to mediate the execution of apoptosis. We demonstrated that caspase-3 was cleaved upon *Y. pestis* infection in wild type but not in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.11A). However, the recent report by Philip et al. indicates that *Y. pseudotuberculosis*-induced cell death is not affected in caspase-3<sup>-/-</sup> or caspase-7<sup>-/-</sup> macrophages (FigS1, Philip et al., 2014), suggesting that caspase-3 and caspase-7 may play redundant roles to regulate apoptosis downstream of caspase-8, or that caspase-8 may have alternative substrates. Deleting both caspase-3 and caspase-7 simultaneously will address the question of whether these caspases play redundant roles.

RIP3 functions as the critical regulator in necrosis (see Chapter I). Here we show that *Yersinia*-induced cell death is intact in RIP3<sup>-/-</sup> macrophages, suggesting that necrosis is not the preferred method by *Yersinia* to kill cells. In a surprising contrast to RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages, *Y.*

*pestis* still induced obvious cell death in caspase-8 conditional knockout cells (CKO, caspase-8 fl/fl LysM cre<sup>+/+</sup>) (Fig. 2.10CD). Although there is still a small amount of caspase-8 expression remaining in the CKO cells (Fig. 2.10A), the difference between RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> and caspase-8 CKO cells suggests that RIP3 can be also involved in *Y. pestis*-induced cell death. In caspase-8 deficient dendritic cells, LPS alone can promote RIP3-mediated necrosis (Kang et al., 2013). Through the use of genetically different mouse models, it is well accepted that caspase-8 blocks RIP3-mediated necrosis during normal embryogenesis and when caspase-8 is deficient, RIP3-induced necrosis will be unblocked to cause embryonic lethality (Kaiser et al., 2011; Oberst et al., 2011). To investigate whether the death of caspase-8 CKO macrophages is mediated by RIP3, we pretreated cells with specific RIP3 kinase inhibitor GSK'872. As expected, this RIP3 kinase inhibitor significantly reduced the death of caspase-8 CKO but not WT macrophages (Fig. 2.10D). Moreover, pretreatment with the pan-caspase inhibitor zVAD-fmk completely inhibits *Yersinia*-induced cell death in RIP3<sup>-/-</sup>, but not WT macrophages (data not shown; Philip et al. 2014: Fig. 2C). These results suggest that *Y. pestis* induces RIP1-caspase-8-mediated apoptosis under normal conditions and that RIP3 is not required for YopJ-induced cell death in the presence of caspase-8. However, when caspase-8 is lacking or is chemically inhibited, RIP3-mediated necrosis will be unblocked to act as an alternative option to eliminate the infected cells (schematic model Fig. 3.1). The occurrence of necrosis in caspase-8 CKO macrophages still needs to be confirmed either by morphology (electron microscopy) or by immunostaining with a necrosis-specific antibody, like anti-phospho-MLKL (see Chapter I).

Both caspase-8 cleavage and activation induced by *Y. pestis* were absent in RIP1<sup>-/-</sup> fetal liver macrophages (Fig. 2.7A, C), suggesting that RIP1 is required for the proteolytic cleavage and activation of caspase-8. It has been proposed that the catalytic activity of caspase-8 may play a role in the suppression of necrosis through cleaving RIP1 and RIP3 (Chan et al., 2003; Feng et al.,

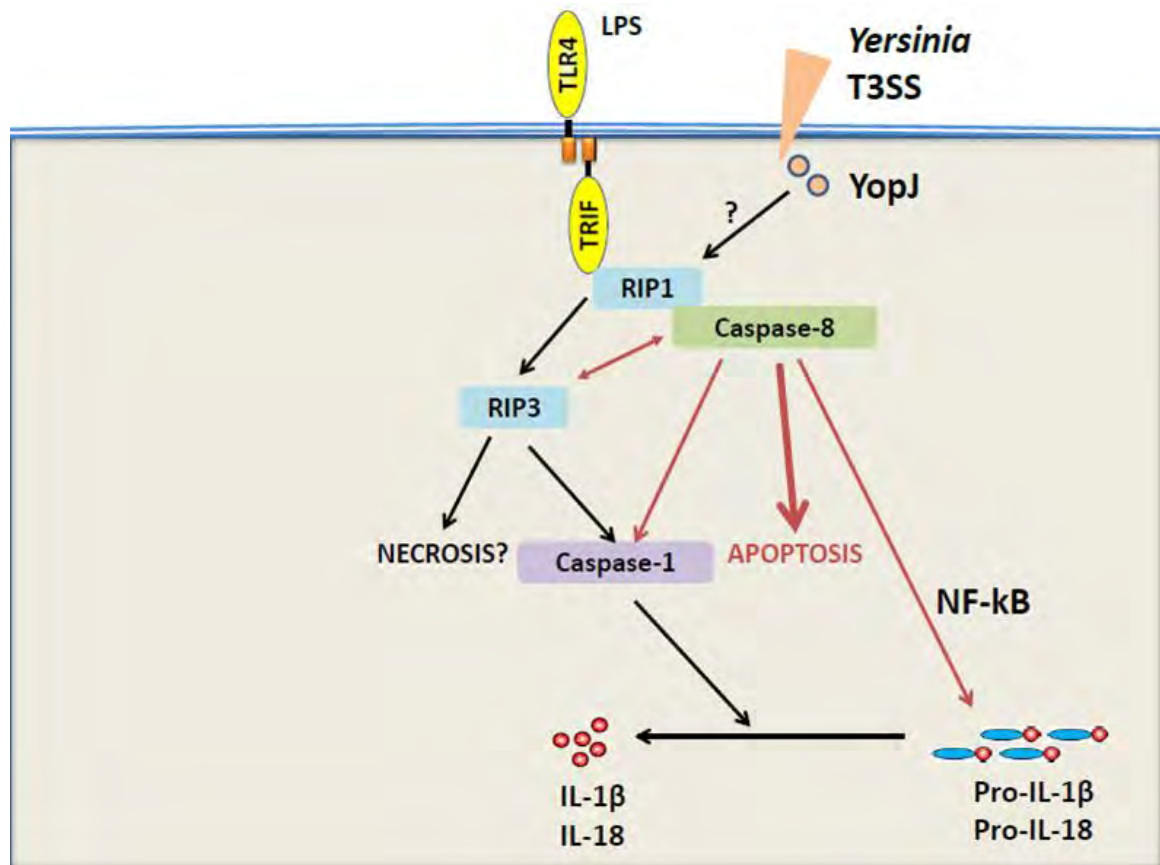


Figure 3.1 Schematic model of *Y. pestis*-induced cell death and innate immune responses.

2007; Oberst et al., 2011). Our results indicate that a portion of RIP1 is cleaved by *Y. pestis* in WT but not caspase-8 deficient cells (Fig. 2.10E). These results imply a mutual interplay between RIP1 and caspase-8, and that they may influence the cleavage and activation of one another. Since the cleavage of caspase-8 is known to be caused by auto-proteolysis, we propose that *Y. pestis* infection stimulates the formation of RIP1-caspase-8-FADD complex, which brings the caspase-8 into their own proximity and allows its activation and auto-proteolytic cleavage. The active caspase-8 thereby acts on RIP1 to induce its cleavage. This hypothesis is somewhat supported by the RIP1 kinase inhibitor GSK'963 which inhibits caspase-8 activation induced by *Y. pestis* (Fig. 2.7B), since that the kinase activity of RIP1 is important for the ripoptosome assembly (Tenev et al., 2011). Although it is still possible that RIP1 can directly target caspase-8 to induce its cleavage and activation, the underlying mechanism and the interplay between RIP1 and caspase-8 need further investigation.

## **NF- $\kappa$ B activation**

Apart from the contribution to *Y. pestis*-induced cell death, RIP1 and caspase-8 are also involved in NF- $\kappa$ B activation and the optimal production of NF- $\kappa$ B-mediated cytokines: IL-6, TNF $\alpha$  and pro-IL-1 $\beta$  (Fig. 2.12). RIP1 is known to regulate the NF- $\kappa$ B pathway when stimulated by death receptors, TLR3 and TLR4 ligands in a kinase-independent manner. The mechanism of RIP1 mediating the TNF $\alpha$ -induced NF- $\kappa$ B activation has been described earlier in this chapter. For mediating the NF- $\kappa$ B activation by TLR3 or TLR4 ligands, RIP1 binds to TRIF via a homotypic RHIM domain interaction and is thereafter ubiquitinated to recruit TAK1 for downstream NF- $\kappa$ B signaling. Caspase-8 has also been reported to mediate NF- $\kappa$ B activation by LPS, antigen receptors, viral infections or Gram-negative bacteria such as *E. coli* and *Salmonella* (Su et al.,

2005; Man et al., 2013; Kang et al., 2008). However, unlike RIP1, both our results and data from Su et al. indicate that caspase-8 is not required for TNF $\alpha$ -mediated NF- $\kappa$ B activation (Fig. 2.13D. Su et al., 2005). This suggests that there might be discrepancy in the mechanism of RIP1- and caspase-8-regulated NF- $\kappa$ B pathway dependent on cellular context and stimulation. The underlying mechanism of caspase-8-mediated NF- $\kappa$ B activation is currently unclear. However, enzymatic activity but not auto-processing of caspase-8 has been suggested to be essential to mediate NF- $\kappa$ B activation (Su et al., 2005). Caspase-8 may regulate the NF- $\kappa$ B pathway through directly targeting its specific substrates. Potential candidates include cFLIP and Cyldromatosis (CYLD). cFLIP (cellular FLICE-like inhibitory protein) is an enzymatically inactive paralogue of caspase-8, with three isoforms: cFLIP<sub>s</sub> (cFLIP short), cFLIP<sub>L</sub> (cFLIP long) and cFLIP<sub>r</sub> (cFLIP Raji) (Irmeler et al., 1997; Yu and Shi, 2008). Caspase-8 is able to cleave cFLIP<sub>L</sub> at Asp376 to generate N-terminal p43FLIP, which interacts with TRAF2, Raf1 and RIP1 to augment NF- $\kappa$ B activation (Kataoka and Tschopp, 2004; Koenig et al., 2014). CYLD is a de-ubiquitinating enzyme that negatively regulates NF- $\kappa$ B signaling by cleaving K63 polyubiquitin chains from RIP1, TAK1, TRAF2 and NEMO (Christofferson et al., 2014). Active caspase-8 is able to cleave CYLD at a conserved site, Asp215, and mutation of this caspase-8 cleavage site on CYLD promotes necroptosis instead of NF- $\kappa$ B activation (O'Donnell et al., 2011). Meanwhile, our results indicate that the I $\kappa$ B degradation pattern is similar between RIP1<sup>-/-</sup> and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.13C, Fig. 2.14B), and that only the later time points of I $\kappa$ B degradation is impaired in the knockout cells. This suggests that the late phase of NF- $\kappa$ B activation mediated by TRIF rather than MyD88 downstream of TLR4 is affected. Furthermore, cytokine production induced by the TLR2 ligand Pam3Cys was unaffected in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages (Fig. 2.12AB). Since TLR2 utilizes MyD88/Mal not TRIF to transduce signaling, thus, caspase-8 might mediate NF- $\kappa$ B activation through cooperation with RIP1 to affect the TRIF signaling arm.

These potential mechanisms are not mutually exclusive, but thorough investigations into the detailed mechanism of caspase-8-mediated NF- $\kappa$ B activation are still required.

## Inflammasome and caspase-1 activation

This work also reveals a striking role of caspase-8 and RIP1 to mediate the processing of caspase-1. The cleavage of caspase-1, which is normally induced by inflammasome activation, is completely blocked in RIP1<sup>-/-</sup> fetal liver macrophages and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMDMs during *Y. pestis* infection (Fig. 2.16CD). Active caspase-1 is responsible for the maturation and secretion of IL-1 $\beta$  and IL-18. Accordingly, we also observed that IL-1 $\beta$  and IL-18 production induced by *Y. pestis* was significantly reduced *in vitro* and *in vivo* when caspase-8 and RIP3 are deficient (Fig. 2.15AB, Fig. 2.18CD). We cannot currently exclude the possibility that caspase-8 also directly cleaves pro-IL-1 $\beta$  independent of caspase-1, as suggested for TLR ligands (Maelfait et al., 2008), Fas ligand (Bossaller et al., 2012), fungi and mycobacteria sensed by dectin-1 (Gringhuis et al., 2012). However, our western blot results suggest that caspase-8 mediates the processing of caspase-1 to regulate the maturation of IL-1 $\beta$  cytokine (Fig. 2.16). This phenotype has also been observed in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> BMDMs treated with the endoplasmic reticulum (ER) stress-inducing drugs plus LPS (Shenderov et al., 2014). Both caspase-1 cleavage and IL-1 $\beta$  release induced by *Salmonella* or Pam3Cys plus nigericin are not affected in RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> or RIP1<sup>-/-</sup> macrophages, suggesting that these knockout cells preserve the capability to undergo inflammasome activation and RIP1 and caspase-8 are not universally required for caspase-1 processing or IL-1 $\beta$  release. The underlying mechanism of RIP1 and caspase-8-mediated caspase-1 processing is unknown. Our results implicate that TLR4-TRIF axis, which may participate to act upstream of caspase-8, is not absolutely required (Fig. 2.17DE). Similarly, TLR4 and TRIF

are required for caspase-8-mediated caspase-1 cleavage and IL-1 $\beta$  production induced by LPS stimulation in the context of ER stress (Shenderov et al., 2014). Philip et al. reconstituted immortalized RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> macrophages with either WT caspase-8 or mutant caspase-8 lacking the three critical aspartate residues required for auto-processing (D3A Caspase-8) and demonstrated that WT caspase-8 but not the D3A caspase-8 reconstitution restored *Y. pseudotuberculosis*-induced caspase-1 cleavage (Fig. 2G of Philip et al., 2014). The D3A caspase-8 mutant is not only uncleavable, but also lacks catalytic activity, suggesting that the caspase-1 processing ability of caspase-8 may be attributed to its enzymatic activity (Philip et al., 2014). This is consistent with our result that the caspase-8 enzymatic inhibitor, IETD, blocks the cleavage of caspase-1 induced by *Y. pestis* (Fig 2.16A). Additionally, the depletion or genetic deficiency of cIAPs not only induces cell death via the formation of ripoptosome as discussed earlier in this chapter, but also triggers IL-1 $\beta$  release via NLRP3-caspase-1- or caspase-8-dependent pathways (Vince et al., 2012). As previously shown, *Y. pestis* is able to induce cIAP degradation (Fig. 2.11C), which may cause the caspase-8 activation and thereby mediate caspase-1 processing and IL-1 $\beta$  release.

In contrast, caspase-8 has also been shown to block RIP1-RIP3-mediated NLRP3 inflammasome activation by LPS in dendritic cells (Kang et al., 2013). LPS alone induces high IL-1 $\beta$  production in caspase-8 deficient but not WT dendritic cells, through inducing RIP1-RIP3-dependent NLRP3-ASC inflammasome assembly (Kang et al., 2013). It is uncertain whether caspase-8 inhibits RIP1-RIP3-facilitated inflammasome activation through directly targeting the inflammasome components, or the deletion of caspase-8 disturbs the cellular homeostasis and thereby indirectly initiates the activation of inflammasome. These studies suggest that caspase-8 represents both stimulatory and inhibitory roles to regulate inflammasome activation and IL-1 $\beta$

production, depending on stimuli and context. Multiple mechanisms might contribute to the complicated role of caspases-8 in regulation of the inflammasome activation.

Our results also suggest that ASC is involved in caspase-8-mediated caspase-1 processing, as caspase-1 cleavage is absent in ASC<sup>-/-</sup> BMDMs (Fig. 2.17F, Zheng et al., 2011). IL-1 $\beta$  production induced by *Y. pestis* is also significantly reduced in ASC<sup>-/-</sup> macrophages (Vladimer et al., 2012; Zheng et al., 2011). *Salmonella* or *Francisella tularensis* infection is able to induce the recruitment of caspase-8 to ASC containing specks (Man et al., 2013; Pierini et al., 2012). Moreover, caspase-8 has been shown to directly interact with ASC through binding to the pyrin domain of ASC (Pierini et al., 2012; Sagulenko et al., 2013), further suggesting that ASC might contribute to caspase-8-mediated caspase-1 cleavage. However, the role of ASC in caspase-8-mediated caspase-1 processing and IL-1 $\beta$  maturation is still debated. Philip et al. observed the caspase-1 cleavage induced by *Y. pseudotuberculosis* YopJ is independent of ASC. Shenderov et al. also indicates that ASC is dispensable for caspase-8-mediated IL-1 $\beta$  maturation induced by ER stress plus LPS. The reason for this discrepancy of the role of ASC in *Yersinia* infection is not clear, but may be due to the different activities of YopJ in *Y. pestis* compared to *Y. pseudotuberculosis* or the different infection conditions.

### **The role of caspase-8 and RIP kinases during *in vivo* *Y. pestis* infection**

Our *in vitro* work indicates that caspase-8 and RIP kinases play critical roles in *Y. pestis*-induced cell death and innate immune responses, including caspase-1 processing, IL-1 $\beta$  maturation and optimal NF- $\kappa$ B activation, all of which are important to counteract pathogen infection. To expand these results *in vivo*, we utilized a mouse model of plague. RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice quickly



succumbed to subcutaneous infection with *Y. pestis*, indicating that caspase-8 and RIP3 are key factors to counteract bubonic plague (Fig. 2.18B). In agreement with our *in vitro* results, the death of splenic myeloid cells and the release of cytokines including IL-1 $\beta$ , IL-18, IL-6 and TNF $\alpha$  are significantly reduced in the infected RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice compared to the WT control. As causative agent of plague, *Y. pestis* induces three different forms of diseases via different infection routes, bubonic, septicemic and pneumonic plague (see Chapter I). It will be interesting to investigate whether caspase-8 and RIP3 also play protective roles in the model of pneumonic plague similar to the bubonic disease. Similar to our results, Philip et al. also showed that mice lacking caspase-8 and RIP3 in the hematopoietic compartment are susceptible to oral infection by *Y. pseudotuberculosis* (Philip et al., 2014), which broadens the role of caspase-8 and RIP3 to fight off infection with other human pathogenic *Yersinia* strains.

RIP1 demonstrates similar functions as caspase-8 during *in vitro Yersinia* infection. Presumably, the role of RIP1 *in vivo* should be similar as caspase-8 as well. Although RIP1<sup>-/-</sup> mice are not embryonically lethal, they die shortly after birth, making it difficult to directly test the phenotype of RIP1 deficient mice by *Yersinia* infection (Kelliher et al., 1998). However, our results indicate that RIP1 kinase activity is important to regulate *Yersinia*-induced cell death and caspase-1 processing, which were inhibited by specific RIP1 kinase inhibitor GSK'963 or Nec-1 (Philip et al., 2014). Very recently, several groups have successfully generated RIP1 kinase-dead mice with either a D138N mutation or a K45A mutation (Berger et al., 2014; Kaiser et al., 2014; Newton et al., 2014). All of these mutant mice are healthy and viable. It will be very interesting and informative to infect these RIP1 kinase-dead mice with *Y. pestis* and investigate the *in vivo* role of RIP1 kinase activity.

As described in Chapter I, the contribution of YopJ to the virulence of *Y. pestis* is still controversial. *In vitro*, YopJ acts as a serine/threonine acetyltransferase, and harbors multiple activities, such as inhibiting NF- $\kappa$ B and MAPK pathways, inducing rapid apoptotic cell death, and triggering inflammasome activation. Despite these robust effects *in vitro*, YopJ does not have an equivalent effect *in vivo*. The deletion of YopJ fails to affect the virulence of *Y. pestis* via the subcutaneous infection route (Lemaître et al., 2006). The reason why YopJ exhibits such different effects *in vitro* and *in vivo* is unknown. One possible explanation is that during the disease progression, pathways effected by YopJ counteract each other and therefore the virulence outcome is not significantly influenced. Although YopJ can inhibit NF- $\kappa$ B and MAPK activation and dampen the production of pro-inflammatory cytokines like IL-6 and TNF $\alpha$ , it is able to induce inflammasome activation and prompt the release of IL-1 $\beta$  and IL-18. Therefore, the reduced innate immune effect by decreased IL-6 and TNF $\alpha$  is compensated by the pro-inflammatory effect of IL-1 $\beta$  and IL-18. Meanwhile, cell death induced by YopJ will also trigger inflammation. The RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice or RIP1 kinase-dead mice can be used to dissect the different effects of YopJ *in vivo*, as the cell death and caspase-1 activation induced by YopJ are inhibited in these mutant mice. Through infecting RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice with WT or  $\Delta$ YopJ *Y. pestis*, or infecting WT and RIP3<sup>-/-</sup>caspase-8<sup>-/-</sup> mice with *Y. pestis*  $\Delta$ YopJ strain, more knowledge will be gained regarding the virulence regulation by YopJ.

## Conclusion

*Y. pestis* is well known as an expert pathogen to evade innate immune surveillance to cause plague. Upon infection, *Y. pestis* can replicate quickly in the host to reach extraordinary densities in the blood and tissues, up to 10<sup>8</sup> CFU per milliliter. Disease progresses fast and the patients

succumb to infection before developing an effective adaptive immune response to fight the bacteria. Hence, the innate immune responses are critical to control *Y. pestis* infection, enabling *Y. pestis* to be an ideal model to study the role of innate immune components in bacterial infection. To counteract or escape the recognition by the innate immune system of mammalian host, *Y. pestis* has evolved with different virulence factors, including type three secretion system (T3SS), non-stimulatory LPS at 37°C and so on. Producing non-stimulatory LPS enables the bacteria not recognized by TLR4/MD2; while the T3SS-injected Yop effectors interrupt intracellular signaling pathways, including the inhibition of NF- $\kappa$ B pathway and induction of cell death by YopJ. Our work implies that effects by these virulence factors converge on the axis of RIP1-caspase-8-RIP3 in the host immune cells. Caspase-8 and RIP kinases not only mediate YopJ-dependent cell death, but also regulate the optimal production of pro-inflammatory cytokines like IL-6 and TNF $\alpha$  via NF- $\kappa$ B. Last but not least, RIP1 and caspase-8 are involved in caspase-1 activation, leading to the maturation of IL-1 $\beta$  and IL-18. All these contributions determine the importance of caspase-8 and RIP kinases to combat against bacterial infection *in vivo*. In addition to the well-known pattern recognition receptors, our work strongly suggests that caspase-8 and RIP kinases are critical innate immune components to control bacterial infection. It will be interesting and rewarding to investigate the role of caspase-8 and RIP kinases during infection with other pathogens. The novel roles unveiled in this thesis work not only help broaden our understanding about caspase-8 and RIP kinases, but also provide new insight into designing therapies to treat *Yersinia* infections.

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